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The Immunoregulation of T cell function by CNS endothelium

By

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Abstract

The presence of inflammatory cells within the central nervous system (CNS) including the retina is characteristic of disease states such as human uveitis and multiple sclerosis, both $CD4^+$ T cell mediated immune inflammatory diseases. Normal CNS is devoid of inflammatory cells and is maintained by the specialized nature of the isolated microenvironments. The blood brain and blood retinal barriers (BBB, BRB) composed of highly specialized endothelial cells physically protect the CNS by regulating immune cell entry. The CNS is actively protected by immune privilege in which the local environment is able to suppress potentially devastating immune reactions, through the local expression of FasL and production of cytokines such as TGF-β. Additionally a role for a group of compounds called Statins has been described in the prevention of leukocyte migration from blood vessels to the sites of atherosclerotic lesions.

This study aims to determine potential mechanisms by which microvascular endothelial cells (EC) of the CNS are able to regulate T lymphocyte function, and whether these mechanisms are implicated in disease. The effects of statins on an inflammatory disease of the CNS were also investigated.

An *in vitro* model of the BBB was used to investigate the immunomodulatory effects of EC upon T cells. A transwell insert system was employed to investigate the effects on T cells of co-culture with EC whilst preventing cell-to-cell contact and also the effect of transmigration through EC monolayers. Studies were also undertaken to investigate potential mechanisms by which this regulation may occur.

The effect of statins was investigated using two models of Experimental Autoimmune Uveoretinitis (EAU) as a model of human posterior Uveitis. EAU was induced using SAg peptide or IRBP peptide and the effects of daily administration of statin were assessed using Fluorescein Angiography. Retinal sections were also examined by classical histology. The effects of statins were also investigated using an *in vitro* model of the BRB, time lapse microscopy and flow cytometry. The effects of statins on T cell function were assessed *ex vivo* by splenocyte proliferation assays.

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Abbreviations

aa	Amino acid
Ab	Antibody
AC	Anterior chamber
ACAID	Anterior Chamber associated immune deviation
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
Ag	Antigen
AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
ATP	Adenosine triphosphate
AU	Anterior Uveitis
BBB	Blood brain barrier
BEC	Brain endothelial cells
BRB	Blood retinal barrier
BSA	Bovine serum albumin
C_{15}	Farnesyl isoprenoid
C_{20}	Geranylgeranyl isoprenoid
C3-transferase	*Clostridium Botulinum* C3-transferase toxin
CAAX	Cysteine-Aliphatic aa-Aliphatic aa-Any aa motif
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freunds Adjuvant
CFSE	Carboxy Fluorescein Succinimidyl Esterase
Ci	Curie
CO_2	Carbon Dioxide
Con A	Concanavalin A
CNS	Central Nervous system
CVO	Circumventricular organs
bp	base pairs
BSA	Bovine Serum Albumin

DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune Uveitis
EC	Endothelial cell
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Eth D-1	Ethidium homodimer-1
ELISA	Enzyme linked immunosorbent assay
FA	Fluorescein Angiography
FAK	Focal adhesion kinase
FA	Fluorescein Angiography
Fas L	Fas ligand
FCS	Foetal Calf Serum
FA	Fluorescein Angiography
FITC	Fluorescein isothiocyanate
FPP	Farnesyl pyrophosphate
GA	Glatiramer Acetate
GAP	GTPase activating protein
GEF	Guanine nucleotide Exchange Factor
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine diphosphate
GPP	Geranyl pyrophosphate
GTP	Guanosine triphosphate
HBSS	Hanks Balanced Salts Solution
HEV	High endothelial venule
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IFN-γ	Gamma Interferon
Ig	Immunoglobulin

IgG	Immunglobulin G
IL	Interleukin
IL-2R	Interleukin-2 receptor
IONO	Ionomycin
IU	Intermediate Uveitis
IRBP	Interphotoreceptor binding protein
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
LFA	Leukcoyte function antigen
nm	nanometers
M	Molar
mAb	Monoclonal antibody
MAGUK	Membrane-associated guanylate kinase
MBP	Myelin basic protein
MCP	Monocyte chemotactic protein
ME	Mercaptoethanol
μg	microgram
MIP	Monocyte Inhibitory Protein
MHC	Major histocompatability complex
mL	millilitre
μM	micromolar
mM	millimolar
MMP	Matrix metalloproteinase
MMPI	Matrix metalloproteinase inhibitor
mRNA	messenger Ribose nucleic acid
MS	Multiple Sclerosis
MTB	Mycobacterium Tuberculosis
NEAA	Non-essential amino acids
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PI	Propidium Iodide
PLN	Peripheral lymph nodes
PMA	Phorbol myristate acetate
PNS	Peripheral Nervous system
PPMS	Primary progressive MS
PRMS	Primary relapsing MS
PTX	Pertussis Toxin
PU	Posterior Uveitis
RAPA	Rapamycin
REC	Retinal endothelial cells
RNA	Ribose nucleic Acid
RT	Room temperature
RPE	Retinal Pigment Epithelium
RPMI	Roswell Park Memorial Institute
RRMS	Relapsing remitting MS
SA	Streptavidin
SAg	Retinal Soluble antigen
SA-PE	Streptavidin-Phycoerythrin
SA-TR	Streptavidin-Texas Red
S.D.	Standard Deviation
SDF	Stromal Derived Factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- PAGE
SEM	Standard error of the mean
sFasL	soluble FasL
SLO	Scanning laser opthalmoscope

SP	Sodium pyruvate
SPMS	Secondary progressive MS
TCR	T cell receptor
TEM	Transendothelial migration
TGF-β	Transforming growth factor- beta
TJ	Tight junction
TNF-α	Tumor Necrosis Factor-α
TNF-R	Tumour Necrosis Factor receptor
TUNEL	Terminal dUTP nick end labeling
v/v	volume to volume ratio
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
w/v	weight to volume ratio
ZO	Zonula occludin

Chapter 1

General Introduction

1.1 The Immune System

The immune system is the term given to the complex, intricate network of cells and chemical messengers, which defend the human body against infection and disease. Immunity, a state of protection, is achieved by the close interaction of the innate and adaptive immune system. The innate or "non-specific" immune system is present at birth and generally functions as the first line of defence against infection. Innate immunity is achieved by the presence of physical barriers including the skin and mucous membranes but also by cells of the immune system including macrophages, neutrophils, eosinophils and basophils. In contrast the adaptive or "specific" immune system, responds to antigens in a highly regulated manner. Re-exposure to an antigen results in a rapid and highly specific immune response, which develops by the process of immunological memory.

The adaptive immune system is mediated by two main cell types, T and B lymphocytes. T and B lymphocytes also known as T and B cells generate immunity by two separate but interconnected processes. B cells mediate humoral immunity through the generation of highly specific soluble receptors termed antibodies (Ab). Binding of an antibody to its specific antigen acts as a signal to other cells of the immune system for its removal. In contrast to B cells, T cells only recognise antigen via the T cell receptor (TCR) when it is complexed to a self polypeptide molecule termed the major histocompatibility complex (MHC) which is expressed by antigen presenting cells (APC) such as dendritic cells (DC). The full proliferative response of a naïve T cell is only achieved upon additional ligand-receptor interactions in a process termed co-stimulation (Schwartz 1992). Stimulation of T cells is therefore restricted to sites such as the lymph nodes and spleen where DC's, which express self-MHC and co-stimulatory molecules, reside (Steinman 1991).

Cellular immunity is generated by two functionally distinct subsets of T cells, these are $CD4^+$ T cells which recognise antigen in the context of MHC class II molecules and generally function as T helper cells to amplify the immune response while $CD8^+$ T cells recognise antigen in the context of MHC class I molecules and generally

cytotoxic. Many T cell effector functions are achieved through the production of pro-inflammatory, anti-inflammatory or regulatory cytokines, which help to amplify or modulate the immune response.

1.2 Immune Surveillance

Memory T cells specific for a given antigen are present at low numbers circulating throughout the body. In an attempt to ensure activation of the required T cell in response to antigen-bearing APC, lymphocytes circulate from the blood to the lymphoid system via high endothelial venules (HEV) of the afferent lymph vessels (Marchesi and Gowans 1964, Girard and Springer 1995). Once within the lymphoid system, comprised of lymph nodes and the spleen, T cells which encounter antigen are sequestered within the lymphoid organs where they are thought to divide and differentiate to become effector cells. In contrast, T cells which do not encounter antigen regain access to the bloodstream via the efferent lymphatic system. This process therefore ensures widespread distribution of T cell receptor specificities throughout the immune system.

1.3 The immune system within the CNS

The central nervous system (CNS) including the retina is protected from the normal immune system at the level of the blood vessels by the presence of specialized endothelia characterised by tight intercellular junctions, which form the blood-brain (BBB) and blood-retinal (BRB) barriers. Where once it was thought that the CNS was devoid of immune surveillance, it is now accepted that immune surveillance of the CNS by activated lymphocytes occurs, albeit at a low level (Hickey *et al* 1991). However, it is recognised that the CNS may be adapted to prevent adverse immune responses such as suppression of antigen presentation by the low-level expression of MHC class II (Zamvil and Steinman 1990) and a higher threshold for antigen-elicited immune responses (Matyszak and Perry 1996, Matyszak 1998).

There are two major subgroups of glial cells within the CNS: these are the macroglia such as astrocytes, oligodendrocytes and ependymal cells and the microglia.

Astrocytes, the most abundant glial cell, are thought to play a role in the maintenance of the BBB which is proposed to be mediated either by direct contact with the endothelium (Janzer and Raff 1987) or through secretory factors (Arthur *et al* 1987). Astrocytes have been shown to express MHC class I and class II antigen in response to stimulation (Fierz *et al* 1985, Fontana *et al* 1986) and also have the capacity to secrete cytokines. Oligodendrocytes are responsible for myelin formation within the CNS, while microglia are considered the resident macrophage of the brain (Sedgwick *et al* 1993). It is thought that microglia may play a role in the inflammatory reaction within the CNS because of their phagocytic ability, release of proteinases, the production of oxidative radicals, expression of MHC antigens upon activation, ability to act as APC and also to respond to cytokine.

Immune-mediated inflammatory disorders of the CNS and retina such as multiple sclerosis (MS) and posterior uveitis are characterised by an increased infiltration of leukocytes to these sites due to a compromised barrier. Subsequent activity of leukocytes within the CNS is thought to contribute to the onset and progression of disease and numerous studies have demonstrated the presence of pro-inflammatory cytokines within the CNS. During diseases such as MS, IL-1, IFN-γ, IL-2, TNF-α and TNF-β have been shown to localise to areas of MS plaques (Hofman *et al* 1986, Traugott and Lebon 1988, Selmaj *et al* 1991, Hofman *et al* 1989) and increased levels of IL-6 have been observed within the CNS during an experimental model of MS, experimental autoimmune encephalomyelitis (EAE; Maimone *et al* 1991, Gijbels *et al* 1990). Production of these cytokines has been attributed to activated T cells and macrophages which infiltrate the CNS and also to resident astrocytes and microglia.

1.4 Cytokines and the CNS

The cytokine family is a complex network of small proteins which are generally produced during the effector phase of an immune response and function to regulate inflammatory responses. Pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-2, IL-6, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) play an important role in inflammatory diseases of the CNS such as MS and posterior uveitis,

while modulatory cytokines such as transforming growth factor-β (TGF-β) and IL-10 are known to play a role in the normal homeostasis of the CNS. The effects of some of these cytokines will now be discussed.

1.4.1 TNF-α

TNF-α is produced by activated macrophages, T cells, astrocytes and microglia. Shown to influence EC by increased permeability (Brett *et al* 1989), increased expression of cell adhesion molecules (Pober *et al* 1986) and increased immune cell adhesion to EC (Pohlman *et al* 1986) all of which when combined, facilitate transendothelial migration of leukocytes across the BBB. TNF-α may also play a role in the upregulation of MHC molecules on astrocytes and microglia and thereby increase the capacity for antigen presentation within the CNS. It has also been shown that antibodies blocking TNF-α inhibit the development of EAE, an effect that may be the result of blocking the ability of TNF-α to upregulate adhesion molecule expression by EC (Fabry *et al* 1994; Hughes *et al* 1988).

1.4.2 IFN-γ

IFN-γ is characteristically produced by both $CD4^+$ and $CD8^+$ T cells as a result of activation. It upregulates MHC class I expression on astrocytes, oligodendrocytes and microglia (Hirayama *et al* 1985, Suzumura *et al* 1986, Wong *et al* 1984) and MHC class II expression upon astrocytes, microglia, pericytes, RPE cells and EC (Fabry *et al* 1994, Jørgenssen *et al* 2001). IFN-γ production correlates with increased encephalitogenicity and may contribute to disease by upregulating adhesion molecules on EC, thereby facilitating migration of lymphocytes into the CNS (Dittel 2000).

1.4.3 IL-1

IL-1 is produced predominantly by activated macrophages but also by EC, B cells, microglia and astrocytes in response to stimulation. As a major co-stimulatory cytokine for T cell activation it allows antigen stimulated T cells to proliferate. It is

also thought to potentiate the immune response within the CNS by the induction of other inflammatory mediators such as prostaglandin and collagenase, by an increase in EC leukocyte adhesion and the induction of further pro-inflammatory cytokines such as TNF-α (Chung and Benveniste 1990) and IL-6 (Benveniste *et al* 1990). IL-1 has been shown to play a role in EAE (Jacobs *et al* 1991).

1.4.4 IL-6

IL-6 produced by immune cells including monocytes and macrophages and also EC, is a pleiotropic cytokine, which is able to act in a pro- and anti-inflammatory manner. Readily induced by TNF-α, IFN-γ and IL-1, IL-6 has been shown to stimulate production of acute phase proteins such as fibrinogen and C-reactive protein (Castell *et al* 1988). IL-6 is known to play a role in the activation of T cells and macrophages but is also known to play a key role in the differentiation of B cells to immunoglobulin-secreting plasma cells and may therefore contribute to the production of antibodies within the CNS.

1.4.5 TGF-β

TGF-β is a multifunctional, hormone-like peptide that controls cell proliferation and differentiation of fibroblasts (Lyons and Moses 1990) but shown to inhibt proliferation of epithelial cells (Barnard *et al* 1988). It has since been recognised that TGF-β also elicits highly immunosuppressive effects upon T and B cell proliferation, maturation of cytotoxic T cells and is capable of inhibiting the effects of TNF-α. TGF-β is produced locally within the eye, at levels far in excess of that determined in the periphery. The production of TGF-β is now thought to be closely associated to a subset of T cells deemed regulatory T cells (Wahl *et al* 2004).

1.4.6 IL-10

IL-10 is produced by cells of the monocyte and macrophage lineage and has been shown to demonstrate immunosuppressive effects within the CNS and periphery (Fabry *et al* 1994). Induction of IL-10 has previously been shown to correlate with

the down regulation of pro-inflammatory cytokines during EAE and recovery from disease. More recently production of IL-10 has been associated with functional regulatory T cells (Jonuleit *et al* 2000).

1.5 Immune privilege

Peter Medawar, in 1948, first described the phenomenon of immune privilege as the "protection against rejection conferred upon foreign tissue when grafted to a particular site". This he hypothesised from observations upon the longevity of survival of allogeneic or xenogeneic transplants to immune privileged sites such as the eye, testis or brain and surmised was a result of a lack of lymphatic drainage and physical isolation of the organ by means of a cellular barrier, such as the BRB or BBB, and thus evasion from the immune system.

Since these initial findings, it is now thought that the phenomenon of immune privilege is not simply due to physical isolation of these organs. Research has shown that tissue grafts to immune privileged sites are capable of initiating an antigen-specific immune response but are able to endure a greater survival time (Kaplan and Streilein, 1977) and the discovery of extensive lymphatic drainage in immune privileged sites such as the testis (Neaves and Billingham, 1979) provided some evidence as to the complexity of the generation and maintenance of immune privileged sites.

Immune privileged sites and tissues have since been characterised and the ability to accept foreign grafts is now thought to be due to multiple features. The ability of immune privileged sites or tissues to accept foreign grafts is no longer thought to be simply due to the presence of isolating cellular barriers and lack of efferent lymphatics. Various other factors have also been identified as contributing factors that, to a lesser or greater extent confer immune privilege. This includes the direct drainage of fluid into the blood stream, generation of an immunosuppressive microenvironment by the local production and secretion of cytokines such as TGF-β (Wilbanks and Streilein, 1991, Cousins *et al* 1991), the presence of neuropeptides

such as α-melanocyte stimulating hormone and vasoactive intestinal peptide (Taylor and Streilein, 1994), soluble and membrane bound inhibitors of complement (Shimada, 1970 and Bora *et al* 1993), the presence of intratissue barriers i.e. tight junctions in Sertoli cells and retinal pigment epithelial cells, reduced or absent MHC expression secretion of corticosteroids and Fas ligand expression (FasL; Bellgrau *et al* 1995, Griffith *et al* 1995, Bechmann *et al* 1999).

Current research suggests that the CNS and retina are constantly surveyed for foreign antigen by activated T cells (Hickey *et al* 1991). Although these cells are able to enter the CNS, several mechanisms are thought to prevent T-cell-initiated immune responses. Two known mechanisms of T cell downregulation within the CNS are the presence of downregulatory cytokines and the induction of apoptosis (Pender *et al* 1993). Evidence suggests the induction of apoptosis, as a mechanism of immune privilege, is crucial for the protection of the delicate microenvironment of the eye and brain for the preservation of organ function (Griffiths *et al* 1995).

1.5.1 Anterior chamber associated immune deviation (ACAID)

Immune privilege within the eye is an active immunoregulatory process that is thought to be achieved in part by the induction of a deviant form of systemic immunity in response to antigens present in the eye (Streilein 1987). ACAID is a stereotypic antigen-specific systemic immune response to eye-derived antigens that is characterised by the selective deficiency of Th1 type antigen-specific delayed hypersensitivity and B-cell-derived complement-fixing antibodies. ACAID is observed when a variety of antigens including viruses, corneal allografts, tumour cells and hapten-derived spleen cells are introduced into the anterior chamber (Streilein *et al* 1997) and relies upon the eye-spleen axis to remain anatomically intact for 4-5 days post inoculation (Streilein and Niederkorn 1981, Niederkorn and Streilein 1982). Eyes of EAU animals are unable to support the induction of ACAID, which is believed to be due to the disruption of important parameters of immune privilege, which may include breakdown of the blood-ocular barrier and loss of an

immunosuppressive microenvironment (Ohta *et al* 1999). Regulatory T cells have also been shown to influence the induction of ACAID (Sonoda *et al* 2001).

1.6 The Blood-Brain Barrier (BBB)

The defining feature of the CNS is the presence of the blood-brain barrier (BBB) or blood-retinal barrier (BRB) formed by endothelial cells (EC) which functions to protect the CNS from harmful substances in the blood stream whilst allowing the passage of nutrients essential for CNS function. The existence of a BBB was first described by Ehrlich in the 19th century. He observed that water soluble dyes given peripherally were unable to permeate to the CNS while Goldmann in 1909 showed that these dyes if injected into the CNS stained only the brain and CSF.

1.6.1 Structure of the BBB

Like peripheral capillaries, EC form a cylindrical shape to allow blood flow through the centre of the cylinder or the lumen of the capillary. These EC are highly specialised for this function and can be characterised by the presence of tight continuous intercellular junctions between cells (Reese *et al* 1971; Figure 1.1). The tight junctions (TJs) provide the BBB with a high electrical resistance and help to limit paracellular diffusion. The presence of TJs also results in the formation of a polarised endothelium, which is capable of displaying different cell surface molecules at the luminal (blood) and abluminal (brain) surface. EC of the BBB possess no fenestrations, few vesicles and no fluid-filled bulk transport channels, which again aid to prevent transendothelial transport. The BBB is also able to exclude large molecules, neurotransmitters and toxins as a result of forming a physical barrier and by lacking the typical transport mechanisms that operate in blood vessels in other regions of the body. However EC of the BBB possess higher mitochondrial content than in peripheral tissues (Oldendorf *et al* 1977), which is suggestive of EC energy-dependent transcapillary transport systems. It is known that the BBB is absent from a small region of the brain called the circumventricular organs (CVO), which is an area which functions to release neurosecretory products into the blood stream. However substances, which enter the CNS via the CVO, are restricted from the

extended brain by the presence of specialised ependymal cells called tanycytes, which impede diffusion to the rest of the brain and cerebrospinal fluid (CSF; Maness *et al* 1998). EC forming the BBB are surrounded by a number of accessory structures, which are thought to regulate the development of the BBB. These include pericytes, perivascular macrophages, astrocytes and the basal lamina. Evidence suggests that EC placed in the brain develop characteristics of EC of the BBB (Stewart and Wiley 1981).

1.6.1.1 Pericytes

Pericytes are a group of functionally and structurally heterogeneous cells, which cover approximately 20-30% of the microvascular circumference (Frank *et al* 1987) and are completely embedded in the basement membrane (Graeber *et al* 1989; Figure 1.1). These cells are thought to regulate EC proliferation, survival, migration and differentiation (Hellstrom *et al* 2001), are known to be phagocytic and therefore considered as a second line of defence preventing transport into the CNS parenchyma (Cancilla *et al* 1972, Broadwell and Salcman 1981) and also play a role in the immune response within the CNS (Fabry *et al* 1993, Angelov *et al* 1998).

1.6.1.2 Perivascular Macrophages

Perivascular macrophages (PVM) are a subset of CNS macrophage, which line the vasculature of the CNS except that of capillaries (Mato *et al* 1986). PVM are phagocytic cells which express MHC class I and II (Hickey *et al* 1992), and as such are thought to be capable of antigen presentation.

1.6.1.3 Astrocytes

The BBB is almost completely surrounded by astrocyte foot processes (Figure 1.1), and it is thought that astrocytes may induce BBB properties in brain EC (Cancilla and DeBault 1983, Goldstein 1988) without contributing to the physical barrier of the BBB (Brightman and Reese 1969). Co-culture of astrocytes with human umbilical

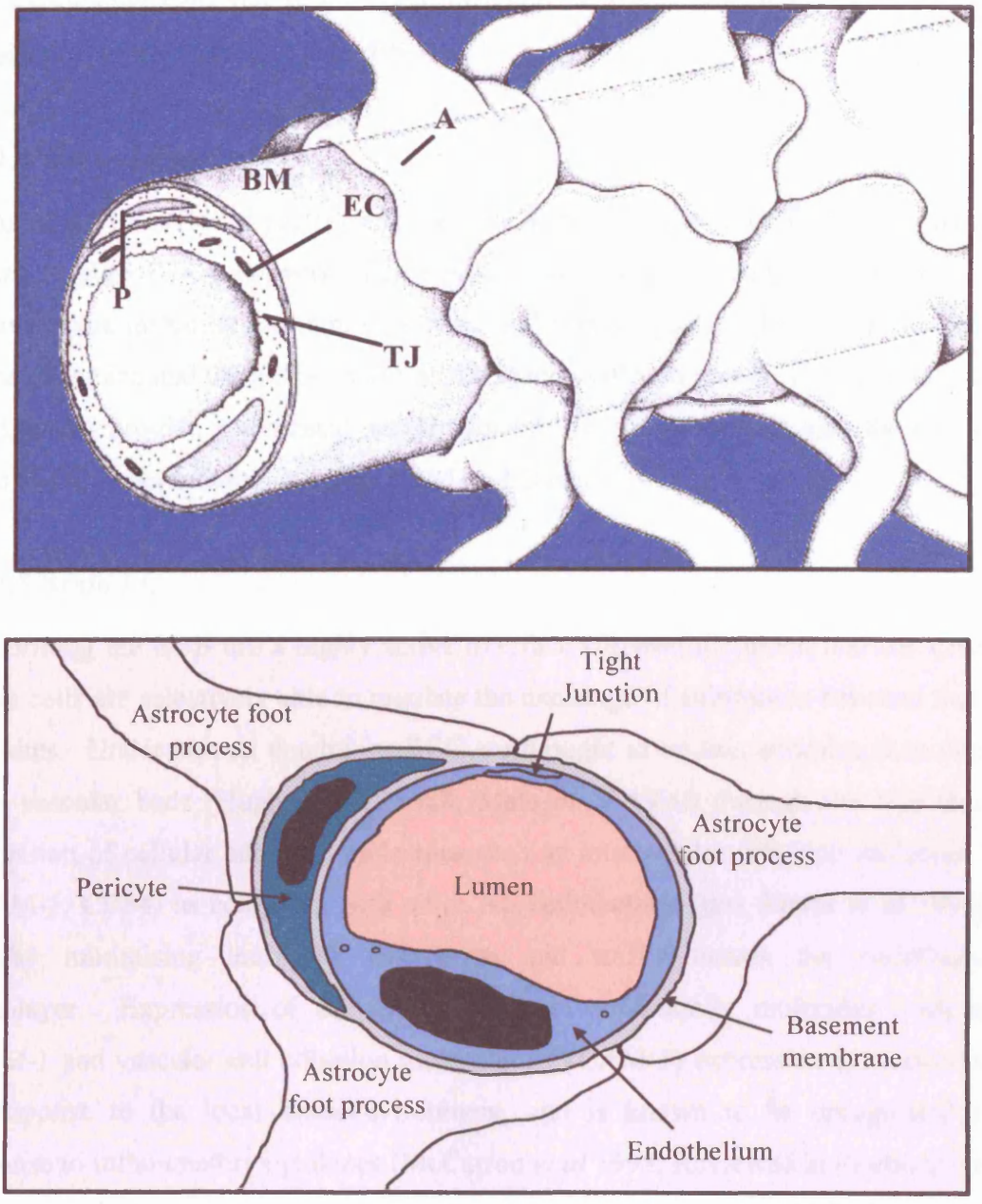

Figure 1.1 Capillaries of the BBB.
Cerebral capillaries of the BBB are lined by specialise EC that lie on a basal lamina in which are embedded pericytes and perivascular macrophages. Abbreviations: P; pericyte, BM; basement membrane (basal lamina), TJ; tight junction, A; astrocyte. Adapted from Bradbury 1985.

vein endothelial cells has been shown to result in a decrease in EC permeability (Rubin *et al* 1991, Hurst and Fritz 1996).

1.6.1.4 The basal lamina

Surrounding the EC and pericytes is the thin basement membrane termed the basal lamina (Figure 1.1), a network of fine protein filaments consisting of a number of glycoproteins including laminin, collagens, and proteoglycan. The region between the basal lamina and the EC is known as the Virchow-Robin space. The basal lamina functions to provide mechanical support for the EC, however it is also thought to regulate EC differentiation, migration and proliferation.

1.6.1.5 Brain EC

EC forming the BBB are a highly active interface between the blood and the CNS. These cells are selectively able to regulate the exchange of substances between these two sites. Under normal conditions BEC are thought to be less adhesive than non-CNS vascular beds (Hughes *et al* 1988, Male *et al* 1990) through the low level expression of cellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1, CD54) as compared with non-CNS endothelium (dos Santos *et al* 1996), thereby minimising leukocyte interaction and traffic across the endothelial monolayer. Expression of the immunoglobulin superfamily molecules such as ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) expression is modulated in response to the local microenvironment, and is known to be upregulated in response to inflammatory cytokines (McCarron *et al* 1993, Reviewed in Roebuck and Finnegan, 1999). CNS EC are also known to express low levels of MHC antigens, which explains their poor ability to support T cell proliferation (Pryce *et al* 1989, Wang *et al* 1995). However, expression is known to be upregulated in response to pro-inflammatory cytokines (Male *et al* 1987), which suggests that CNS EC are capable of a pro-inflammatory role during inflammation.

1.6.1.6 Retinal EC (REC)

REC of the retinal vascular endothelium form the anterior blood-retinal barrier (BRB). REC which are thought to share numerous properties with BEC of the BBB, are known to be in direct contact with circulating leukocytes and are thus thought to mediate control of leukocyte extravasation. Entry into the retina involves leukocyte transmigration across the BRB characterised by the presence of tight junctions. REC have been found to be poor at binding lymphocytes (Hughes *et al* 1999, Male *et al* 1990, Wang *et al* 1993) and under normal conditions are thought to be low expressors of MHC class II. However an enhanced expression of MHC class II is associated with the onset of EAU (Liversidge and Forrester 1988) and ICAM-1 can be induced upon REC in response to IFN-γ (Liversidge *et al* 1990).

1.6.1.7 Retinal Pigment Epithelium (RPE)

Cells of the retinal pigment epithelium form the posterior BRB. In contrast, to the vascular endothelium the RPE lies beyond the vasculature within the cellular architecture of the eye. It is therefore thought that leukocyte recruitment at the posterior BRB is in part regulated by the choroidal endothelium as a result of cytokine influence from the RPE (Devine *et al* 1996). During the course of EAU the RPE is thought to be activated and is induced to express MHC class II antigens (Chan *et al* 1986) and has been shown to be capable of antigen presentation as a result of trypsin treatment of cells (Liversidge *et al* 1993).

1.6.2 BBB/BRB Physiology

Permeability of an endothelium is influenced by a number of factors, however one key factor is the intercellular junctions between EC which gives rise to the measure of electrical resistance. EC of the BBB have been reported to exhibit extremely high electrical resistance of between 1500-2000 ohms per squared cm (Ω cm^2) which is in contrast to values of 3-33 Ω/cm^2 for that of non-CNS endothelia (Crone and Christensen 1981, Butt *et al* 1990). Several other factors, which influence BBB permeability, include specialised transport and enzymatic systems.

1.6.2.1 Endothelial Junctions

Endothelial permeability is in part regulated by intercellular junctions, complex structures formed by adhesive transmembrane molecules which are linked to a network of cytoplasmic or cytoskeletal proteins within the cell. At least four different types of endothelial junctions have been described these are, tight junctions, gap junctions, adherens junctions and desmosomes. Each type of junction is characterised by the expression of specific junctional proteins (Dejana *et al* 1995) and has a specific role in junctional permeability. Expression of the different forms of junctions is thought to be spatially isolated in epithelial cells, however this is not thought to be true for endothelial cells (Schulze and Firth 1993).

1.6.2.1.1 Desmosomes

Desmosomes are localized patches that hold two cells tightly together. They are common in epithelia such as the skin and are linked to the intermediate filaments of keratin in the cytoplasm.

1.6.2.1.2 Gap junction

Gap junctions are intercellular channels composed of a single protein of the connexin family which permit the free passage of ions and small molecules, up to a molecular weight of about 1000 Daltons between the cells (Beyer 1993). These junctions permit changes in membrane potential to pass from cell to cell.

1.6.2.1.3 Adherens junction

Located at the basal side of the EC, adherens junctions are junctions formed by the interactions of cadherins. Cadherins are single chain transmembrane glycoproteins which exhibit homophilic calcium dependent binding (Takeichi 1991, Kemler 1993). The intracellular domain of cadherin is linked to the actin cytoskeleton via a network

of proteins called catenins (Tsukita *et al* 1992, Kemler 1993). It is thought that adherens junctions play a role in cell contact inhibition.

1.6.2.1.4 Tight junction (TJ)

Tight junctions or the zonula occludens are the close connections between epithelial and endothelial cells that generally comprise CNS endothelia. The majority of our understanding about tight junctions comes from studies of epithelia. The TJ consists of three integral membrane proteins, namely, claudin, occludin, and junction adhesion molecules, and a number of cytoplasmic accessory proteins including ZO-1, ZO-2, ZO-3, cingulin, and others (Figure 1.2).

Claudins-1 and -2 were first identified as integral components of the TJ, in 1998, by Furuse *et al.* At least 24 members of the claudin family have since been identified and have been shown to bind homotypically to claudins on adjacent endothelial cells to form the primary seal of the TJ (Furuse *et al* 1999) and to bind to ZO-1, ZO-2, and ZO-3 intracellularly (Furuse *et al* 1999). Occludin was identified as the first integral protein localized at the TJ (Furuse *et al* 1993, Ando-Akatsuka *et al* 1996). Occludin is thought to contribute to the generation of electrical resistance given that the two extracellular loops of occludin and claudin, although originating from neighbouring cells, form the paracellular barrier of the TJ (Hirase *et al* 1997, Sonoda *et al* 1999). Occludin expression is greater in BEC as compared to non-neural tissues. The third TJ-associated membrane protein is the junctional adhesion molecule (JAM; (Martin-Padura *et al* 1998). Three JAM's have been identified (Palmeri *et al* 2000, Aurrand-Lions *et al* 2000) all of which belong to the immunoglobulin superfamily.

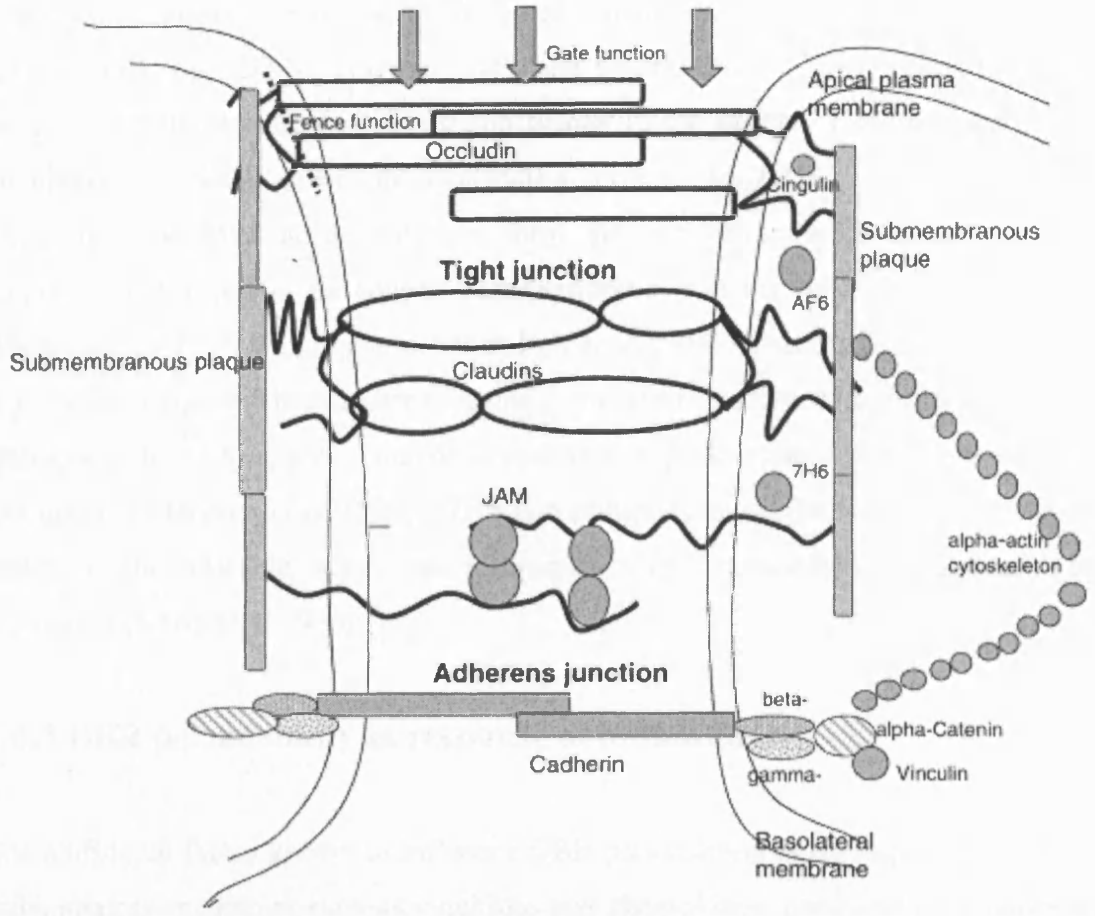

Figure 1.2 Proposed interactions of junctional complex proteins in EC of the BBB.

At tight junctions, claudins have been proposed to function as the backbone by binding to claudins on adjacent cells and thus forming a seal. Claudins also bind to occludin, which functions as a regulatory protein. Occludin's presence in the membrane correlates with increased electrical resistance across the membrane and decreased permeability. Occludin is thought to be involved also in the fence function of tight junctions. JAM is involved in cell–cell adhesion and contributes to permeability control. A series of cytoplasmic proteins ZO-1, ZO-2 and ZO-3 form the submembraneous plaque of tight junctions. These proteins belong to the MAGUK family (membrane-associated guanylate kinase proteins) and are involved in the coupling of transmembrane proteins to the cytoskeleton actin. Several cytoplasmic accessory proteins have been identified at tight junctions, including AF6, 7H6 and cingulin. The adherens junctions are composed of transmembrane glycoproteins of the cadherin super family, which are linked to the cytoskeleton via cytoplasmic anchor proteins, β-catenin, γ-catenin (plakoglobin) and $p120^{ctn}$ that belong to the Armadillo protein family. β-catenin and γ-catenin bind to α-catenin, which is an actin-binding and actin-bundling molecule linking the adhesive cadherin/catenin complex to the F-actin-based cytoskeleton. Taken from Petty and Lo (2002).

Cytoplasmic proteins involved in TJ formation include zonula occludens proteins (ZO-1, ZO-2, and ZO-3), cingulin, 7H6, and several others. ZO-1, -2, and -3 have sequence similarity with each other and belong to the family of proteins known as membrane-associated guanylate kinase-like protein (MAGUKs; Anderson 1996). These proteins have been shown to form the submembraneous plaque of tight junctions and function to couple transmembrane proteins with the cytoskeleton (Haskins *et al* 1998, Fanning *et al* 1998, Itoh *et al* 1999, Ebnet *et al* 2000). Cingulin is a phosphoprotein which localises to the cytoplasmic surface of tight junctions and interacts with ZO-1, -2 and -3 and as a result is thought to act as a scaffold at the tight junction (Cordenonsi *et al* 1999). 7H6 is a phosphoprotein found at tight junctions which are impermeable to ions and macromolecules however its role remains to be elucidated (Satoh *et al* 1996).

1.6.3 BBB permeability in response to inflammation

One additional factor known to influence BBB permeability is the exposure of EC to inflammatory mediators such as cytokines and chemokines, produced by leukocytes of the immune system and also by CNS resident cells.

1.6.3.1 Cytokines

Although cytokines play numerous roles, a key role for cytokines during inflammation within the CNS is their effect upon BBB permeability. Pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1 and IL-6, produced by both immune cells and glial cells, are thought to play a major role in inflammation within the CNS (Benveniste, 1992). *In vitro* treatment of rat cerebral EC (RCEC) with TNF-α, IL-1β and IL-6 resulted in a significant decrease in electrical resistance (de Vries *et al* 1996), while *in vivo* administration of TNF-α, IL-1β and IL-6 has also been shown to result in increased permeability of the BBB (Wright and Merchant 1994, Claudio *et al* 1994, Saija *et al* 1995).

1.6.3.2 Chemokines

Chemokines (chemotactic cytokines) are a family of small-secreted polypeptides, which function to recruit leukocytes to sites of infection and inflammation, including the CNS. Many chemokines are known to be produced during an inflammatory reaction and can be produced by EC in response to cytokines such as TNF-α and IL-1. Chemokines are known to play a major role in the control of leukocyte adhesion, chemotaxis and activation (Eugenin and Berman 2003) and have also been shown to influence the expression of tight junctional proteins such as ZO-1 and occludin, thereby influencing BBB permeability (Song and Pachter 2004).

It is possible to classify chemokines both structurally and functionally. Structural classification of chemokines broadly reveals four families based on the configuration of cysteine residues near the NH_2-terminus of the amino acid structure. The largest family consists of two cysteine residues adjacent to each other and are termed CCL chemokines while CXCL chemokines have two cysteine molecules separated by an intervening amino acid. Two smaller families consist of one member each, these are the XCL family possessing only two cysteine residues and the CX_3CL family with three intervening amino acids between cysteine residues (Campbell and Butcher 2000).

Functional classification of chemokines reveals two main types of chemokines. These are the constitutive and inducible chemokines, although a small number of chemokines exhibit functions of both groups and are therefore considered to have dual-function (Moser *et al* 2004). Constitutive chemokines also known as homeostatic or lymphoid chemokines, are proposed to play a role in the regulation of lymphocyte migration under normal conditions. These are known to be produced in discrete microenvironments within lymphoid or non-lymphoid tissue such as the skin and mucosa and play a role in the positioning of cells of the adaptive immune system during haematopoiesis, antigen sampling in secondary lymphoid organs and immune surveillance of healthy peripheral tissues. In contrast, inducible chemokines also called inflammatory chemokines, are generally expressed during periods of infection,

tissue injury or inflammation and comprise most of the chemokine family. Inducible chemokines produced in inflamed tissues by both resident and infiltrated cells are thought to play a role in the recruitment of effector cells such as monocytes, granulocytes and activated T cells. Dual function chemokines have been shown to predominantly act upon T lymphocytes, playing a role in homing during development and also recruitment during an inflammatory response (Moser *et al* 2004).

Chemokines elicit their action via high-affinity seven-transmembrane spanning G-protein coupled receptors which are classified by means of the chemokines which they recognise followed by R for receptor, namely CCR, CXCR, XCR, CX3CR and a number in order in which they were identified. Several second messengers of G-protein coupled receptors are known to be activated as a result of chemokine receptor ligation including, phospholipase Cβ (PLCβ) isoforms, serine-threonine kinases, phosphatidylinositol 3-kinase-γ (PI3Kγ) and c-Src-related non-receptor tyrosine kinases (Loetscher *et al* 2000, Thelen 2001).

The chemokine network displays a high degree of functional redundancy, in that a given chemokine may be capable of binding several members of the same chemokine receptor family and that one receptor may bind several related chemokines (Rossi and Zlotnik 2000). In general, inflammatory chemokines and receptors tend to participate in overlapping and redundant pairing, whereas those involved in homeostatic homing tend to show more exclusive interactions (Moser and Loetscher 2001; Table 1.1). Mutations in homeostatic chemokine genes are therefore thought to be more detrimental, as has been demonstrated using the *plt* mouse whereby a mutation in the SLC gene results in a lack of lymphocyte homing to secondary lymphoid organs (Nakano *et al* 1997).

One main function of chemokines is the chemotaxis of receptor expressing cells toward higher levels of chemokines. Chemokines have also been implicated in lymphocyte differentiation and effector functions (Luther and Cyster 2001). However chemokines have recently been shown to play a key role in the regulation of

Chemokine Receptor	Chemokine(s) bound	Functional Classification
CXCR1	CXCL2 (GROβ, MIP-2α), CXCL3 (GROγ, MIP-2β), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8).	Inducible
CXCR2	CXCL1 (GROα, MGSA), CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8.	Inducible
CXCR3	CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC).	Dual
CXCR4	CXCL12 (SDF-1).	Constitutive
CXCR5	CXCL13 (BLC, BCA-1).	Constitutive
CXCR6	CXCL16	Dual
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC-1, CKβ1), CCL15 (HCC-2, Lkn-1, MIP-5), CCL16 (HCC-4, LEC, Mtn-1), CCL23 (MPIF-1, ckβ81).	Inducible
CCR2	CCL2 (MCP-1, MCAF), CCL7, CCL12, CCL13 (MCP-4).	Inducible
CCR3	CCL5, CCL7, CCL8 (MCP-2), CCL13 (MCP-4), CCL15, CCL24 (MPIF-2, eotaxin-2), CCL26 (eotaxin-3, MIP-4α).	Inducible
CCR4	CCL17 (TARC), CCL22 (MDC, STCP1).	Dual
CCR5	CCL3, CCL4 (MIP-1β), CCL5.	Inducible
CCR6	CCL20 (MIP-3α, LARC).	Dual
CCR7	CCL19 (MIP-3β, ELC, ckβ11), CCL21 (6Ckine, SLC, ckβ9).	Constitutive
CCR8	CCL1 (I-309), CCL4 (MIP-1β).	Dual
CCR9	CCL25 (TECK, ckβ15).	Dual
CCR10	CCL27 (CTACK, ILC, ESkine).	Inducible
XCR1	XCL1 (Lymphotactin, ATAC).	Inducible
CX_3CR1	CX_3CL1 (fractalkine).	Inducible

Table 1.1 Chemokine receptors and their chemokines.

GRO, growth-related oncogene; MGSA, melanoma growth stimulatory activity; MIP, macrophage inflammatory protein; PF, platelet factor; ENA, epithelial cell-derived neutrophil-activating factor; LIX, lipopolysaccharide-induced CXC human chemokine; GCP, granulocyte chemotactic protein; CK, chemokine; NAP, neutrophil-activating protein; IL, interleukin; Mig, monokine induced by γ-interferon; IP-10, γ -interferon-inducible protein; I-TAC, interferon-inducible T-cell chemoattractant; SDF, stromal cell-derived factor; BCA-1, B-cell-activating chemokine; BLC, B-lymphocyte chemoattractant; BRAK, breast and kidney expressed chemokine; TCA, T-cell-activation protein; MCP, monocyte chemoattractant protein; MCAF, monocyte chemotactic and activating factor; RANTES, regulated on activation normal T-cell expressed and secreted; MRP, MIP-related protein; HCC, hemofiltrate CC chemokine; Lkn, leukotactin; LEC, liver expressed chemokine; Mtn, monotactin; LCC, liver-specific CC chemokine; TARC, thymus- and activation-related chemokine; DC-CK, dendritic cell chemokine; PARC, pulmonary- and activation-regulated chemokine; ELC, Epstein-Barr virus-induced receptor ligand chemokine; LARC, liver- and activation-induced chemokine; 6Ckine, 6 cysteine chemokine; SLC, secondary lymphoid tissue chemokine; MDC, macrophage-derived chemokine; STCP, stimulated T-cell chemotactic protein; MPIF, myeloid progenitor inhibitory factor; TECK, thymus-expressed chemokine; CTACK, cutaneous T cell-attracting chemokine; ILC, interleukin 11 receptor alpha-locus chemokine; ESkine, embryonic stem cell chemokine; I-309, ATAC, derived from gene names.

leukocyte migration out of the bloodstream in the 'address code' theory of leukocyte homing (Butcher *et al* 1999, von Andrian and Mackay, 2000; further discussed in section 1.7.2).

1.7 Leukocyte migration

Recruitment of leukocytes from the circulation to peripheral tissues is a key event in the development of inflammatory disease including inflammatory diseases of the CNS such as multiple sclerosis (MS) and posterior uveitis. Under normal conditions very few leukocytes are detectable within the CNS (Hickey *et al* 1991), however an increased number of inflammatory cells are present during CNS disease. Leukocyte migration from the bloodstream into the CNS depends upon the interaction of activated leukocytes with EC that form the BBB and the BRB. The process of entry to peripheral tissues is termed extravasation, diapedesis or transendothelial migration (TEM; Figure 1.3). The process of lymphocyte TEM is well characterised and it is known that EC of the BBB play a major role in the regulation of leukocyte entry to the CNS. Leukocyte TEM is also known to be regulated by the leukocyte. A "multistep paradigm" for leukocyte migration was first described by Springer in 1994. This consisted of four sequential stages for leukocyte interaction with the endothelium; these are, leukocyte tethering and rolling, EC signalling to leukocytes, firm adhesion and diapedesis. However it has since been shown that leukocytes are able to signal to the endothelium and therefore there are five main stages involved in leukocyte TEM.

1.7.1 Leukocyte tethering and rolling

The initial stages of the adhesion cascade are mediated by adhesion molecules known as the selectins. These are a family of transmembrane molecules, expressed on the surface of leukocytes and activated EC, which bind to carbohydrates. Selectin-mediated interactions between the EC and leukocyte are rapid and short-lived and result in the characteristic slowing down of circulating lymphocytes and rolling along the surface of the endothelium (Lawrence and Springer 1991). There are three

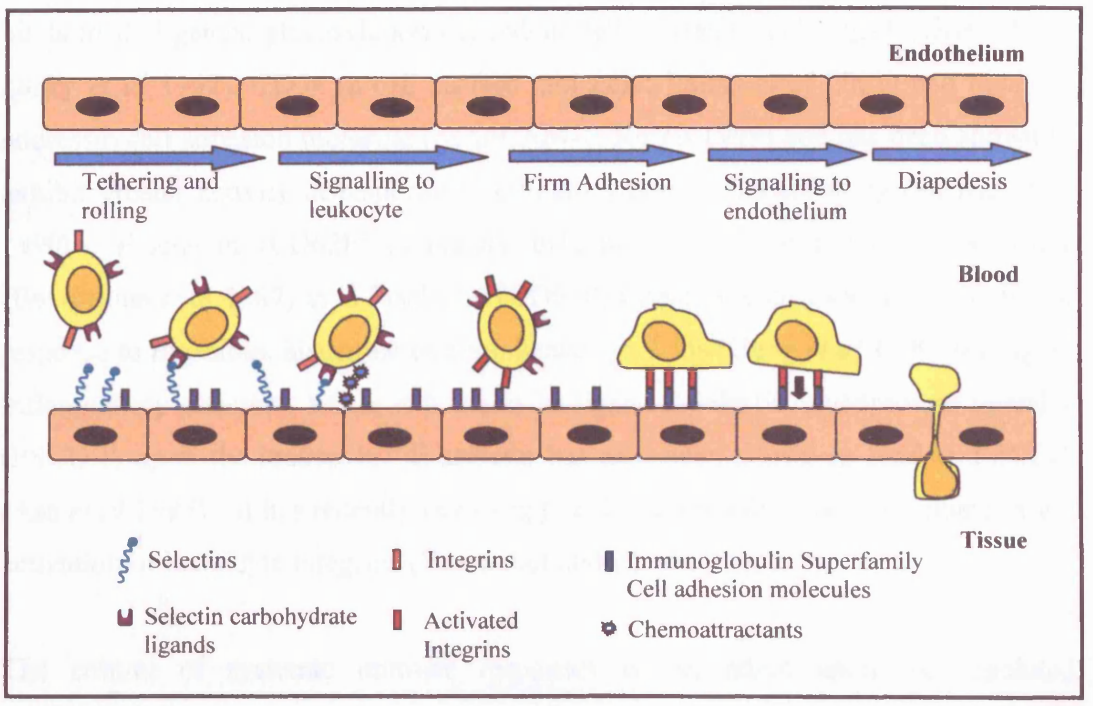

Figure 1.3 Leukocyte TEM.
The leukocyte adhesion cascade is a sequence of adhesion and activation events, which ends with extravasation of the leukocyte, whereby the cell exerts its effects on the local environment. Five main steps are involved in leukocyte adhesion to and migration out of the vascular endothelium: (1) Tethering and rolling, in which selectins and glyco-conjugated selectin-ligands are involved, (2) signalling of the endothelium to the leukocyte, mediated by soluble and/or membrane-bound chemoattractants such as chemokines, (3) Firm adhesion (4) signalling of the leukocyte to the endothelium and (5) Transmigration or diapedesis, which are mediated by integrins and their ligands, e.g. intercellular adhesion molecules.

members of the selectin family: L-selectin (leukocyte-selectin) expressed on all circulating leukocytes except for a subpopulation of memory lymphocytes; P-selectin (platelet-selectin), so-called because it was first observed upon platelets, but now also observed within Weibel-Palade bodies of EC; E-selectin (endothelial-selectin) expressed by inflamed vascular endothelium (Springer 1994). L-selectin (CD62L) binds to its ligands, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1; Lasky *et al* 1992), CD34 (a cell surface molecule; Lanza *et al* 2001) and mucosal addressin cell adhesion molecule (MAdCAM-1; Rosen 1999) and has been shown to exhibit greater activity in response to cytokines prior to its shedding (Griffin *et al* 1990). E-selectin (CD62E) is readily inducible by pro-inflammatory cytokines (Bevilacqua *et al* 1987) as is P-selectin (CD62P) which is readily expressed by EC in response to thrombin, histamine or complement proteins (Geng *et al* 1990) during an inflammatory response, where it binds to its ligand P-selectin glycoprotein ligand-1 (PSGL-1) upon the leukocyte. E-selectin has also been shown to bind to PSGL-1 (Asa *et al* 1995). It has recently been suggested that selectins may participate in the activation of leukocyte integrins (Vestweber and Blanks 1999).

The control of systemic immune responses is dependent upon the regulated trafficking of leukocytes. This is achieved by means of a homing system which is in part facilitated by selectin molecules. In order for an activated leukocyte to interact with an endothelium, leukocytes must express the requisite molecules for recognition of the endothelium. Selectin interactions play a key rold in the first stage of leukocyte TEM during tissue specific lymphocyte homing (Butcher and Campbell 1996). The interaction of lymphcoyte L-selectin with EC PNAd or MAdCAM-1 is responsible for the homing of naïve B and T lymphocytes to peripheral lymph nodes and Peyers patches respectively (Butcher and Picker 1996). Expression of cutaneous lymphcoyte antigen (CLA) and its interaction with E-selectin is known to play a key role in the tissue specific homing of skin homing memory cells to skin (Picker 1994) while $\alpha 4\beta 7$ binding of MAdCAM-1 is thought to play a role in the homing of memory cells to the lamina propria of the small intestine (Berlin *et al* 1995).

1.7.2 Signalling to the leukocyte

Selectin interactions are thought to bring the leukocyte and EC into close proximity and result in the exposure of leukocytes to chemoattractants expressed or secreted by the endothelium as it is proposed that chemokines expressed upon the surface of the endothelium may influence leukocyte migration (Rot 1992, Tanaka *et al* 1993). It has been shown that EC exposed to inflammatory cytokines such as TNF-α and IL-1 produce chemokines such as monocyte chemotactic proteins (MCP's), RANTES, fractalkine and macrophage inflammatory proteins (MIP; Baggiolini 1998, Ben-Baruch *et al* 1995). Chemokines are known to elicit multiple functions including directing the migration of leukocytes however, it has been shown that signalling of chemokines such as stromal derived factor (SDF-1), 6-C-kine and MIP-3β via its respective G-protein coupled receptor expressed upon the leukocyte induces the rapid activation of leukocyte integrins resulting in an increase in integrin avidity for its receptor. This is thought to function as a checkpoint prior to the firm adhesion of reversibly adhered leukocytes (Campbell *et al* 1998). Chemokines, like selectins are thought to play a role in the regulation of tissue-specific lymphocyte homing (Campbell *et al* 1999) at the level of endothelium-leukocyte signalling. The SLC-CCR7 interaction is known to play an essential role in the TEM of naïve T cells to peripheral lymph nodes and has shown to result in the rapid adhesion of T cells to HEV (Warnock *et al* 2000). Homing of skin memory T cells to inflamed skin is known to be mediated by interaction of TARC with its receptor CCR4 (Campbell *et al* 1999) while homing of gut memory T cells to the small intestine lamina propria is elicited by the interaction of TECK with CCR9 (Zabel *et al* 1999). Leukocytes that do not express the requisite chemokine receptors fail to undergo subsequent integrin activation and therefore are unable to mediate firm adhesion to and subsequently migration through the endothelium.

1.7.3 Firm adhesion

Firm adhesion of the leukocyte to the endothelium is mediated by binding of the integrin family of molecules expressed by the leukocyte with their corresponding

ligands, members of the immunoglobulin (Ig) superfamily, expressed on the endothelium. Circulating leukocytes maintain their integrins in a non-adhesive state in order to prevent non-specific attachment to the endothelia of blood vessels. However integrins can readily be activated by the action of endothelium derived chemoattractants such as chemokines. It is this activation of leukocyte integrin molecules, which permits firm adhesion of the leukocyte to the endothelium and subsequently leukocyte transmigration.

A number of integrins are known to play a role in leukocyte-endothelial interactions; these include very late antigen-4 (VLA-4), leukocyte function antigen-1 (LFA-1), Mac-1 and LPAM-1 (lymphocyte Peyer's patch HEV adhesion molecule (Larson and Springer 1990, Stewart *et al* 1995). Several members of the Ig superfamily have been implicated in leukocyte TEM, including ICAM-1 and 2 and also VCAM-1 (Vonderheide and Springer 1992). ICAM-1 is expressed at low levels upon the vascular endothelium and has been shown to be upregulated in response to pro-inflammatory cytokines (Dustin *et al* 1986, Wellicome *et al* 1990). ICAM-1 binds to LFA-1 and has been shown to be essential for TEM into the CNS both *in vivo* (Whalen *et al* 2000) and *in vitro* (Greenwood *et al* 1995), as demonstrated by antibody-mediated blockade of LFA-1 and ICAM-1 mediated antigen-specific T cell migration across primary retinal (Greenwood *et al* 1995) and primary cerebral endothelium (Pryce *et al* 1997). In contrast, ICAM-2, also known to bind LFA-1, is constitutively expressed and is thought to play a role in lymphocyte recirculation in uninflammed tissues (Springer 1994). VCAM-1 is a ligand for VLA-4 and LPAM-1 (Chan *et al* 1992, Ruegg *et al* 1992) and its expression has been shown to be upregulated in response to pro-inflammatory cytokines (Dustin *et al* 1986, Wellicome *et al* 1990).

1.7.4 Signalling to the endothelium

Binding of leukocyte LFA-1 to ICAM-1 upon the EC has recently been demonstrated to result in conformational changes within both the leukocyte and EC in order to permit TEM. It has since been shown that this is as a result of ICAM-1 ligation-

induced signalling pathways, which are thought to play a role in TJ permeability (see chapter 5).

1.7.5 Diapedesis

Diapedesis through the vascular endothelium is the final stage in TEM. It is not entirely clear how leukocytes mediate this stage. However, two possible mechanisms include intercellular transmigration and intracellular transmigration. Intercellular or paracellular migration is the most characterised route for TEM (Johnson-Leger *et al* 2000), although EC junctions are known to express numerous proteins which function to maintain endothelium integrity through the formation of TJ. It has previously been reported that neutrophils migrate through the endothelium at the junction of three EC (Burns *et al* 1997). It has been suggested that leukocyte migration into the CNS results in the activation of signal transduction cascades which lead to the loss of TJ-associated molecules including occludin and ZO-1 and subsequent BBB breakdown (Bolton *et al* 1998). A significant body of evidence exists to support migration directly through EC (Greenwood *et al* 1994, Feng *et al* 1998, Wong *et al* 1999). This is thought to be due to active remodelling of the EC membrane causing invagination and fusion of the luminal and abluminal surface of the EC (Feng *et al* 1998).

1.7.6 Migration through the basement membrane

Once through the endothelium, leukocytes must traverse the basement membrane, which is composed of a network of glycoproteins. Migration at this stage is thought to rely upon a number of proteolytic systems such as heparanase, plasmin and A disintegrin and metalloproteinases (ADAMs). Goetzl *et al* in 1996 demonstrated a clear role for matrix metalloproteinases (MMP's), in particular MMP2 and MMP9, in lymphocyte migration through the basement membrane.

1.8 Leukocyte trafficking during CNS disease

Increased trafficking into the CNS is thought to occur following infection, ischemia, trauma and autoimmunity. Each of these events results in signalling at the vascular endothelium to which immune system cells respond by the process of TEM. Although all cells of the immune system are able to enter in response to inflammation autoimmune inflammation within the CNS as is proposed to occur during MS and uveitis is predominantly due to T lymphocytes and cells of the monocyte/macrophage lineage.

1.8.1 T lymphocytes

All activated T cells express the capacity to migrate across the BBB and BRB independent of antigen specificity and subtype (Hickey *et al* 1991, Wekerle *et al* 1991). Initial activation of T cells is thought to occur in the periphery and it is proposed that antigen recognition within the CNS occurs as a result of molecular mimicry between a microbial antigen and a CNS antigen (Barnaba and Sinigaglia 1997, Hafler 1999), as a result of cross reactivity with bacterial or viral peptides (Goverman *et al* 1993), as a result of activation by bacterial superantigens (Brocke *et al* 1995) or due to cytokines released in response to an unrelated inflammatory reaction (Gutierrez-Ramos *et al* 1992). Activation of the T cells results in an upregulation of cell adhesion molecules, interaction with the CNS endothelium, TEM and entry into the CNS parenchyma. Once within the CNS, T cells are thought to encounter MHC class II^+ perivascular macrophages where it is believed that T cells recognise their specific antigen, which results in the local activation of the T cell, the stimulus for the subsequent inflammatory cascade, and ultimately damage to the CNS. T cell recognition of antigen results in pro-inflammatory cytokine production, which results in the activation of the BBB by an increased expression of ICAM-1 (Matsuda *et al* 1994) and VCAM-1 (Wong *et al* 1999) and induction of chemokine production by astrocytes and microglia (Fuentes *et al* 1995), which combine to further recruit leukocytes into the CNS. Cytokine production by T cells also results in an increased capacity for antigen presentation by resident cells such as astrocytes and microglia of the brain (Benveniste and Benos 1995). T cells are also directly able

to mediate oligodendrocyte death (Jurewicz *et al* 1998, Jewtoukoff *et al* 1989, Selmaj *et al* 1991, Antel *et al* 1994), which in turn results in the characteristic myelin loss observed in MS and EAE. It has been suggested that the chronicity observed in some instances may be as a result of a phenomenon of epitope spreading as new self peptides emerge as a result of tissue damage (Lehman *et al* 1992, McRae *et al* 1995, Tuohy *et al* 1999).

1.8.2 Monocytes and Macrophages

Cells of the monocyte/macrophage lineage comprise a resident population within the CNS in the form of microglial cells and perivascular macrophages (Hickey and Kimura 1988) and, as a result, it is accepted that monocytes enter the CNS in order to differentiate into these cells (Lassmann *et al* 1991). However, during inflammation of the CNS, monocytes are thought to migrate to the CNS, differentiate to become macrophages or microglia and contribute to the destruction of neuronal myelin either by phagocytosis or by the production of cytokines (Bauer *et al* 1994, Ruuls *et al* 1995, Chia *et al* 1983). This is a major factor in the progression of CNS diseases such as MS. CNS microglial cells have been shown to play an important role in the effector stage of EAE (Sedgwick *et al* 1987).

1.9 Immune mediated diseases of the CNS and Retina

A number of disorders are associated with the CNS including Stroke, Alzheimer's Disease, Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency syndrome (AIDS). These diseases may arise as a result of immune dysregulation or immune cell infiltration to these sites as a consequence of the disease. However a group of T cell-mediated autoimmune diseases exists and it is T cell migration across the blood-brain and blood-retinal barriers, which results in the generation of an immunopathological response. Evidence suggests an increased incidence of lymphocyte extravasation to sites such as the brain or retina results in immune-mediated diseases of the CNS such as MS and posterior uveitis.

1.9.1 MS

MS is a chronic, often relapsing condition, which damages the white matter of the CNS, the brain and spinal cord, affecting approximately 85,000 people in the UK alone (MS Society website). First described by Charcot in 1868, MS was the term given to a condition characterised by neurological symptoms and intention tremors which at post mortem revealed disseminated lesions in the white matter of the brain. Several factors have since been shown to influence MS; these include genetics, gender and environmental triggers.

Susceptibility to MS is linked to genes within the MHC complex on chromosome 6 (Haines *et al* 1996, Sawcer *et al* 1996, Ebers *et al* 1996) while epidemiological studies have shown a link between the incidence of MS and distance from the equator. These studies revealed that the incidence of MS is higher in the UK, North America and Scandinavia but virtually non-existent in Malaysia or Ecuador, which supports the role for an environmental trigger in disease (Compston *et al* 1998). The role of the environment is further supported by studies of migrant populations (Dean 1967, Dean and Kurtzke 1971, Kurtzke *et al* 1979).

MS is generally diagnosed between the ages of 20 and 40, is twice as common in females as in males (National MS Society website) and can only be made upon demonstration of two or more attacks affecting two or more areas of the CNS, when all other neurological conditions have been ruled out (McDonald *et al* 2001). Tests for the diagnosis include head and spine magnetic resonance imaging (MRI; Ormerod *et al* 1987), neurological examination, and lumber puncture sampling for the determination of oligoclonal banding of CSF (Lowenthal *et al* 1960, Christenson *et al* 1983).

1.9.1.1 Clinical MS

MS can present in a number of forms and as a result of this symptoms of MS vary from individual to individual. Some common symptoms of MS include sensory problems, visual problems, fatigue, dizziness, pain, loss of muscle strength, problems

with balance and coordination, bladder and bowel problems, cognitive problems and sexual problems. However, as with many other aspects of this multifactorial disease, MS can occur randomly and in an unpredictable manner. It is possible to classify MS disease in one of four major groups- Relapsing remitting MS, Secondary Progressive MS, Primary progressive MS and Primary relapsing MS.

Approximately 80% of patients diagnosed with MS present with symptoms of relapsing remitting MS (RRMS; Compston and Coles 2002). This stage of disease is characterised by defined self-limited relapses or attacks which last for at least 24 hours. Between relapses or attacks patients can make a full recovery and are found to be neurologically and symptomatically "normal" in a period termed remission. An attack or relapse is a period in which patients experience new symptoms or recurrence of old symptoms which vary in their intensity and duration and it is at this time in which inflammatory cells are thought to attack the nerve fibres of the CNS. Secondary Progressive MS (SPMS) is characterised by the steady deterioration in neurological function as a result of significant nerve fibre loss. This stage of disease can present in the presence or absence of isolated attacks. 50% of all MS patients diagnosed with RRMS are thought to progress to SPMS. In contrast Primary progressive MS (PPMS) presents as a disease with a steady decline in function from onset of disease without isolated attacks. Approximately 10% of all patients are diagnosed with this form of MS which predominantly affects the spinal cord. Progressive relapsing MS (PRMS) is the least common form of MS and progressive from onset. Currently 90% of MS patients will progress to a progressive form of disease within 25 years of diagnosis.

1.9.2 Posterior Uveitis

Inflammation of the eye can be caused by a variety of diseases other than bacterial infection, many of which are sight-threatening. These include the group of intraocular immune-mediated diseases, known as uveitis, which is responsible for 10% of all registered blind patients under the age of 65 years. Uveitis is the term

given to any inflammatory condition affecting the uvea which is composed of the iris, ciliary body, and choroid. Causes of uveitis can include autoimmune disorders, infection, or exposure to toxins and the disease presents in many forms. The symptoms and signs of uveitis may be subtle, and vary considerably depending on the site and severity of the inflammation. However in many instances, the cause remains unknown.

1.9.2.1 Clinical uveitis

Uveitis is most commonly classified anatomically as anterior, intermediate, posterior, or diffuse, depending on which segment of the eye is affected. The most common form of uveitis is anterior uveitis (AU), which involves inflammation in the front part of the eye, usually isolated to the iris or the ciliary body. This condition is often called iritis and iridocyclitis and is generally the most symptomatic form of uveitis, generally presenting with pain and redness, photophobia and decreased vision. The inflammation may be associated with autoimmune diseases but in most cases occurs in healthy people and does not indicate an underlying disease. Most common in young and middle-aged people, AU does not generally lead to visual loss. Intermediate uveitis (IU), also called peripheral uveitis, affects the area immediately behind the iris and lens in the region of the ciliary body and pars plana, and may also be referred to as "cyclitis" and "pars planitis". IU is classically painless and presents with floaters and decreased vision. This form of disease predominantly affects teenagers or sometimes young children. Symptoms present as visual blurring without pain or redness and usually affect both eyes.

Posterior uveitis (PU) signifies a number of forms of uveitis affecting the posterior segment of the eye. Inflammation presents in the choroid, retina or the blood vessels as choroiditis, retinitis, vasculitis or optic neuritis. Posterior uveitis is usually painless yet is far more likely to result in visual impairment, and is often associated with numerous other conditions including toxoplasmosis, Behcet's Disease, sarcoidosis and MS. Drops or injections fail to reach the back of the eye and

therefore treatment is generally administered systemically, which means the whole body is affected and may suffer side effects. Pan uveitis is the term given to inflammation involving all parts of the eye, including anterior, intermediate, and posterior structures. Endophthalmitis is a severe, acute, diffuse uveitis resulting from intraocular infection. Pan uveitis may produce any or if affects all of the above-mentioned symptoms and signs. Complications of uveitis may result in profound and irreversible loss of vision, especially when unrecognized or treated improperly with the most frequent complications including cataract; glaucoma; retinal detachment; neovascularization of the retina, optic nerve, or iris; and cystoid macular oedema. This thesis will concentrate upon those clinical forms of uveitis characterised by the presence of cell infiltration of the retina which will be referred to as posterior uveitis.

1.9.2.2 Posterior uveitis pathology

Activated T cells play an important immunopathogenic role in the development of posterior uveitis (Deschennes *et al* 1988). Uveitis is characterised by the predominance of a $CD4^+$ T cell infiltrate which are HLA DR positive (Charteris et al 1992). An upregulation of adhesion molecule expression is thought to occur which, in turn potentiates an increase in lymphocyte TEM (Whitcup *et al* 1992). An upregulation of HLA-DR upon ocular resident cells by has also been demonstrated during uveitis (Chan *et al* 1986, George *et al* 1997), which may contribute to increased antigen presentation to the infiltrating lymphocytes in order to perpetuate the inflammatory cycle.

1.10 Experimental models of CNS disease

1.10.1 Experimental Autoimmune Encephalomyelitis (EAE)

EAE is a $CD4^+$ T cell-mediated autoimmune disease of the CNS which is used extensively and from which much of our understanding of the basic mechanisms thought to occur during MS is derived (Wekerle *et al* 1994). Different EAE models facilitate investigation of different immunopathogenic aspects of autoimmune

neuroinflammation which may have relevance for its human counterpart. EAE has been induced in a number of different animal species including mice, rats, guinea pigs, rabbits, macaques, rhesus monkeys and marmosets, following a protocol of active immunisation with encephalitogenic CNS-derived antigen peptides (Costa *et al* 2003) or via the immunisation of activated encephalitogenic T cells which are able to confer disease in naïve animals (Lublin, 1985).

There are two main models of EAE: acute EAE which is a monophasic disease induced upon immunisation with myelin basic protein in complete Freunds adjuvant (Paterson and Hanson 1969), and chronic relapsing EAE (crEAE). crEAE is characterised by the induction of an acute phase of EAE which is followed by a period of spontaneous remission and subsequent relapses. A more clinically relevant model for MS, crEAE can be induced by two immunisations of spinal cord homogenate in complete Freunds' adjuvant containing *Mycobacterium tuberculosis* H37RA and *Mycobacterium butyricum* one week apart (Baker *et al* 1990, Brown and Moon 1998). A number of myelin components can be used to induce crEAE including MBP, myelin oligodendrocyte glycoprotein (MOG) and proteolipoprotein (PLP).

1.10.1.1 Induction of EAE

EAE can also be mediated by the active immunisation of the dominant encephalitogenic epitope of MOG^{35-55} peptide in C57BL/6 mice (Slavin *et al* 2001, Costa *et al* 2003). Immunisation of animals following this protocol was found to result in clinical onset of disease 10-15 days post immunisation with characteristic infiltration of inflammatory cells.

1.10.2 Experimental Autoimmune Uveoretinitis EAU

Posterior uveitis can be studied using an experimental model of disease called EAU which is a prototypic $CD4^+$ T cell-mediated disease that targets the neural retina and related tissues (Caspi *et al* 1989, Gery *et al* 1985) and is therefore considered a good

experimental model for investigating how T cells cross the BRB, induce retinal inflammation and uveitis. A number of experimental models of posterior uveitis have since been established using a variety of retinal antigens in a number of animal species with models described in the rat, mouse, guinea pig, rabbit and monkey (Caspi 1997). Of note is that species-specific and strain-specific differences exist between different models of EAU and EAE.

1.10.2.1 Retinal Autoantigens

Wacker and Lipton in 1968 determined that the retina contained a number of potent autoantigens and as a result EAU models in the rat and mouse have been developed in response to three main antigens: Retinal Soluble Antigen, Interphotoreceptor Retinoid Binding Protein and Rhodopsin. However it is known that EAU can be induced in response to recoverin, phosducin and even non-retinal antigens such as melanin associated protein and myelin basic protein. Most models of EAU have been established using bovine retinal antigens.

Retinal Soluble Antigen, SAg or Arrestin is a 48kD protein of the retina. Located within the rod outer segments, SAg is involved in the phototransduction cascade and responsible for binding photoactivated-phosphorylated rhodopsin. SAg from retinal rod photoreceptor cells induces a $CD4^+$ T cell mediated autoimmune disease of the retina and uveal tract of the eye and the pineal gland (Chan *et al* 1985). Originally whole SAg was isolated from bovine eyes (Fong *et al* 1984). It has since been shown that synthetic peptides of SAg peptide can mediate EAU (Donoso *et al* 1990). SAg peptide-specific T cells derived from SAg peptide immunised animals have also been shown to be capable of mediating EAU in naïve animals (Fling *et al* 1991). Interphotoreceptor retinoid binding protein (IRBP) is a 148kD protein found within the photoreceptor matrix. IRBP is thought to transport vitamin A between the photoreceptor layer and the RPE. IRBP immunisation of Lewis rats induces severe autoimmune uveoretinitis and pinealitis (Dua *et al* 1992, Gery *et al* 1986). Rhodopsin and its illuminated form, opsin, is the rod cell visual pigment protein. The

pathogenicity of this 40kD protein has been shown to be conformation-dependent (Schalken *et al* 1988).

1.10.2.2 Induction of EAU

SAg-induced uveitis in Lewis rats is a short acute disease (De Kozak *et al* 1981, Mochizuki *et al* 1985). A similar disease can also be induced by immunisation of synthetic peptides of retinal autoantigens such as SAg or IRBP in an antigenic adjuvant, such as Freunds' adjuvant (Fling *et al* 1991, Hankey *et al* 2001). Immunisation with synthetic bovine SAg peptide 273-289 results in the development of EAU and the generation of uveitogenic T cells *in vitro* (Fling *et al* 1991). Uveitogenic or disease-mediating $CD4^+$ T cells can be isolated from PLN from SAg immunised rats following a method described by Mochizuki *et al* (1986). Adoptive transfer of such recently activated uveitogenic $CD4^+$ T cells expressing the molecules necessary for migration have been shown to mediate induction of EAU (Gregerson *et al* 1986, Mochizuki *et al* 1985).

1.11 Therapies for CNS diseases

1.11.1 Therapies for MS

The aims of therapy for MS are to prevent or postpone long-term disability and to alleviate symptoms of acute attack. At present, management of MS generally requires a multidisciplinary approach in order to accommodate the variety of problems an MS sufferer may encounter such as physiotherapy and speech therapy. However a number of disease-modifying drug therapies for MS have been investigated in an attempt to modulate a number of immunological aspects of the disease. As a result, therapies trialled in the treatment of MS have included anti-inflammatory, immunomodulatory and immunosuppressive agents.

Current management of MS is predominantly through the use of glucocorticosteroids such as methylprednisolone and dexamethasone, which are highly potent

immunomodulatory and anti-inflammatory drugs. Steroids have previously been shown to increase recovery speed as a result of an MS relapse but have no impact upon the degree of recovery. Steroids are thought to alleviate symptoms of disease by the reduction of BBB abnormalities (Miller *et al* 1992), a reduction in levels of circulating $CD3^+$ T cells (Dufour *et al* 1994) and decreased leukocyte adhesion molecule expression (Droogan *et al* 1998).

The most common therapy for MS at present is β-interferon (IFN-β). The initial rationale for the use of IFN-β in MS was the belief that the disease may be caused by a latent viral infection within the CNS (Jacobs and Johnson 1994). Various preparations of β-interferon (β-interferon-1a and 1b) have undergone clinical trials and have been shown to reduce relapse rate which is thought to be mediated by the suppression of Th1 type cell generation (McRae *et al* 2000), although effects beyond the reduction in relapse rate are unknown.

Several other therapies have recently been investigated. Glatiramer Acetate (GA) also known as copolymer-1, was first found to inhibit EAE (Teitelbaum *et al* 1971). It has subsequently been shown to reduce relapse rate and disability progression in RRMS (Johnson *et al* 1995, Johnson *et al* 1998). It is thought to cause immune deviation from a Th1 to a Th2 response, induce antigen-specific suppressor T cells, and inhibit antigen presentation (Neuhaus *et al* 2001). Side effects to GA therapy are mild but include transient chest pain and lymphadenopathy (Ziemssen *et al* 2001). Mitoxantrone, an immunosuppressive agent which inhibits T helper cell functions, has been shown to be beneficial in the treatment of disability progression, relapses and the formation of new lesions (Millefiorini *et al* 1997). However, it is thought from other studies investigating the effects of mitoxantrone in Hodgkin's disease that therapy may result in severe side effects (Aviles *et al* 1993). The use of azathioprine and cyclophosphamide in the management of MS predate strict clinical trials and as such their clinical effects are undetermined. Both appear to exhibit high levels of toxicity and therefore serious consideration must be given prior to use. Most recent therapies for MS have involved the development of monoclonal antibodies which are

able to disrupt specific stages of disease which include natalizumab, an antibody which recognises α4 integrins such as VLA-4, and thereby prevents leukocyte migration into the CNS (von Andrian and Engelhardt 2003); or Campath-1, marketed as alemtuzumab, which is a monoclonal antibody which recognises CD52 expressed by all leukocytes and results in leukocyte depletion. Both of these drugs have been subject to clinical trials in MS and have proved very effective (Tubridy *et al* 1999, Coles *et al* 1999) although side effects have been reported for Campath-1 (Coles *et al* 1999).

1.11.2 Therapies for Uveitis

The main aims of uveitis therapy are to relieve pain and discomfort, prevent sight loss due to the disease or possible complications and to treat the causes of disease. If inflammation is caused by viral or bacterial infection then treatment is with anti-virals or antibiotics. However, the cause of uveitis in the majority of cases remains undiagnosed. As a result of this, the immediate aim of treatment is to decrease inflammation and so alleviate symptoms of disease. This is achieved by means of steroids, immunosuppressants and mydriatics.

As with MS, glucocorticosteroids form the first line of defence for the treatment of non-infectious uveitis (Jabs *et al* 2000). This is due to their rapid ability to suppress inflammation through mimicking the function of cortisol (hydrocortisone). For the treatment of uveitis, steroids can be administered topically, systemically or in a peri-ocular manner while in sight threatening cases it is possible to administer intravenous steroids if so required. Steroids used for the treatment of uveitis include prednisone. However, several side effects occur as a result of steroid therapy including nausea, weight gain, fluid retention, hypertension, diabetes and long term therapy has been shown to give rise to severe ocular complications such as cataract and glaucoma. Mydriatics and cycloplegics such as atropine or cyclopentolate are antimuscarinics which cause dilation of the pupil and paralysis of the ciliary muscle, and function to alleviate symptoms of pain and discomfort associated with AU. These drugs vary in

potency and duration of their effects. The major group of compounds used in the treatment of uveitis are immunosuppressants these are often administered in combination with steroids to act in a steroid-sparing manner. Immunosuppressants which are currently used include antimetabolites such as azathioprine (Imuran), methotrexate (Rheumatrex) and mycophenolate mofetil (Cell cept). These have been shown to be effective in the treatment of uveitis and in some instances the treatment of steroid-resistant or steroid-intolerant uveitis (Holz *et al* 1992, Kilmartin *et al* 1998, Lau *et al* 2003, Baltatzis *et al* 2003). Although minor side effects have been reported, mycophenolate mofetil is reportedly successful in the treatment of uveitis with an acceptable profile of side effects (Larkin and Lightman 1999).

Cyclosporine (Sandimmune, Neoral, and SangCyA) an inhibitor of T cells functions by blockade of gene transcription and hence proliferation and cytokine production. It has previously been found to be highly effective in the treatment of a number of different forms of uveitis (Nussenblatt *et al* 1983) with success comparable to that of steroid therapy (Nussenblatt *et al* 1991). Cyclosporine therapy has highlighted side effects which include muscle cramps, nephrotoxicity and elevated blood pressure with a major side effect of suppression of the immune system resulting in an increased rate of infection. The majority of uveitis patients are children, and long term cyclosporine therapy has been shown to be highly detrimental for development (Ellis *et al* 1985). Tacrolimus (FK506; Prograf) is an antibiotic of the macrolide family which inhibits expression of early T cell activation genes and thus inhibits T cell mediated responses by mechanisms similar to cyclosporine (Suzuki *et al* 1997). Previously shown to be highly effective in the treatment of EAU (Kawashima *et al* 1988) and endotoxin-induced uveitis (Whitcup *et al* 1998) it has shown efficacy in the treatment of refractory uveitis (Mochizuki *et al* 1993, Kilmartin *et al* 1998, Sloper *et al* 1999).

Rapamycin (RAPA or sirolimus) like tacrolimus, is an antibiotic of the macrolide family which inhibits the late phase of IL-2 receptor mediated T cell activation (Ikeda *et al* 1997). RAPA was found to be highly effective in the inhibition of EAU

(Roberge *et al* 1993, Ikeda *et al* 1997). However a side effect of RAPA is hyperlipidaemia, including hypercholesterolemia and hypertriglyceridemia (Groth *et al* 1999). Current RAPA therapy may therefore be administered in conjunction with statins, which are inhibitors of the cholesterol synthesis pathway. As a last resort alkylating agents such as cyclophosphamide and chlorambucil have been used in the treatment of sight threatening disease despite severe side effects, which include sterility.

More recently treatment of uveitis has focused upon more specific targets including the use of biological therapies such as etanercept (Enbrel), a fusion protein comprised of the extracellular ligand binding portion of the human TNF-receptor and the Fc portion of human IgG1, which is capable of inhibiting the function of TNF-α and TNF-γ. Conflicting evidence exists for the efficacy of etanercept at present (Reiff *et al* 2001, Reddy and Backhouse 2003). A second anti-TNF-α therapy includes infliximab (Remicade), a chimeric monoclonal antibody which binds to both membrane and soluble TNF-α (Scallon *et al* 1995) with exceptional efficacy documented in rheumatoid arthritis (Maini *et al* 1998, Lipsky *et al* 2000, Elliott *et al* 1993, Elliott *et al* 1994). Infliximab therapy has shown to be successful in a number of forms of uveitis (El-Shabrawi and Herman, 2002, Sfikakis *et al* 2001, Joseph *et al* 2003). A third TNF therapy investigating the ability of the p55 TNF receptor fusion protein (TNFr-Ig) has shown clinical efficacy in the treatment of posterior uveitis (Murphy et al 2004). Campath-1 or alemtuzumab is a monoclonal antibody directed against the CD52 antigen expressed by human lymphocytes. Antibody recognition of $CD52^{+}$ leukocytes results in their death and therefore leukocyte depletion. The antibody is fully humanised and has previously been found to be effective in the treatment of MS, despite some side effects (Coles *et al* 1999). In addition, research has demonstrated clinical efficacy of Campath-1 in refractory ocular inflammatory disease (Isaacs *et al* 1996 and Dick *et al* 2000). Daclizumab (Zenapax) is a humanised monoclonal antibody, which recognises the alpha subunit of the IL-2 receptor (p55 alpha, CD25 or Tac subunit) and inhibits binding of IL-2 to its receptor. It functions by the suppression of IL-2-mediated activation of lymphocytes and has

since been deemed safe for long term use in intermediate and posterior uveitis (Nussenblatt *et al* 1999, Nussenblatt *et al* 2003).

1.12 Potential therapies for CNS disease

It is widely accepted that numerous events are involved between predisposition to disease and expression of disease. It has previously been suggested that events including peripheral activation of CNS reactive T cells, migration of these activated T cells to the CNS, crossing of the BBB and perpetuation of the immune response once within the CNS, represent several stages at which therapeutic intervention may be targeted (Pender and Wolfe 2002).

1.12.1 Statins

Statins or HMG CoA reductase inhibitors are a group of compounds which were originally isolated from Penicillium species by Endo *et al* (1976) but may also be derived from Aspergillus and Monascus. The active compound "compactin" was found to lower cholesterol in normolipidemic and hyperlipidemic individuals. A number of compactin derivatives are now available and have clinical efficacy for the treatment of hyperlipidemia and atherosclerosis. Statins such as lovastatin, simvastatin, pravastatin, and mevastatin are type I statins or naturally occuring statins. Fluvastatin, atorvastatin, cerivastatin, rosuvastatin and pitavastatin are considered type II statins and are synthetic statins. Statin-mediated inhibition of cholesterol synthesis is achieved by the similarity in structure of statins and HMG-CoA. These compounds are based on a lactone ring structure and can exist in a pro-drug form, which must be converted to the active acid form for its biological activity. Hydrolysis of lovastatin to lovastatic acid can produce between 4-6 metabolites.

Statins have been shown to inhibit the activity of HMG-CoA reductase in a competitive, specific and reversible manner. It has been demonstrated that statins inhibit *de novo* synthesis of cholesterol and also increase LDL receptor expression on

the cell surface, which increases catabolism of cholesterol from the circulation and both result in a decrease in circulating cholesterol levels. Statins have also been shown to possess a higher affinity for HMG-CoA reductase (Ki=3.5-44nM) than HMG-CoA itself (Km=4μM; Holdgate *et al* 2003).

Statins can either be completely absorbed after oral administration or require metabolism from the pro-drug form to the active form by the liver. A number of statins have undergone extensive trials for the treatment of hypercholesterolemia and few adverse effects have been reported including liver toxicity and myopathy.

1.12.2 Statin therapy

Atherosclerosis is known to be a multifactorial disease characterised by the complex interaction of lipid metabolism, mononuclear cells, coagulation proteins, cytokines, the extracellular matrix (ECM) and haemodynamic forces (Libby 2000) and as such is suggested to be a chronic inflammatory disease (Vaughan *et al* 1996, Ross 1999, Lusis 2000). The rapid effectiveness by which statins decrease coronary events suggested that statins are able to influence vascular biology independently of lowered plasma cholesterol. It has been suggested that statins influence the blood vessel wall, blood flow and constituents of blood (Vaughan *et al* 1996). However they have also been shown to exert a number of varied effects which include vasodilative, antithrombotic, antioxidant, antiproliferative, anti-inflammatory and plaque stabilising effects (Davingnon and Laaksonen 1999).

1.12.3 Statins in EAE

A number of recent studies have demonstrated the ability of statins to ameliorate EAE. These studies revealed that the effects of statins upon this multifactorial disease are numerous and those known, to date, include the suppression of pro-inflammatory cytokines decreased MHC expression and inhibtion of MMPs (Stanislaus *et al* 1999, Stanislaus *et al* 2001, Youssef *et al* 2002, Greenwood *et al* 2003, Aktas *et al* 2003). Subsequent to these findings in EAE, clinical trials are

underway in order to test statins in the treatement of MS and preliminary reports suggest a high degree of clinical efficacy (Vollmer *et al* 2004).

1.12.4 Statins in EAU

Given the reported success of statins the treatement of EAE and MS and that similar mechanisms are believed to play a role in the induction of EAU, we investigated whether statins were able to modulate clinical EAU. In subsequent chapters of this thesis, the effects of statins upon EAU will be described. As previously discussed RAPA is proposed to induce an increase in serum cholesterol, thus patients currently undergoing treatment for uveitis may receive statins in the treatment of RAPA-induced hyperlipidemia. It would therefore be extremely interesting to investigate the anti-inflammatory effects of statins in uveitis patients undergoing RAPA therapy.

1.13 AIMS

It has previously been shown that activated T lymphocytes are able to migrate across the BBB and BRB into the CNS and that BEC and REC play a role in the regulation of lymphocyte migration. Therefore I initially set about to investigate the immunoregulatory effects of CNS EC upon T cells as a result of TEM *in vitro*.

Secondly given the role of the EC in the regulation of lymphocyte trafficking into the CNS and the requirement of leukocyte trafficking into the CNS for the initiation of CNS mediated diseases such as uveitis and MS I investigated whether disruption of EC signalling pathways by statins is able to modulate lymphocyte migration into the CNS using both *in vitro* and *in vivo* models.

Chapter 2

Materials and Methods

2.1 Materials

Product	Product Code	Supplier
100 bp DNA ladder	15628-019	Invitrogen UK
2-Mercaptoethanol	M6250	Sigma Laboratories UK
CFSE (CFDA tracker kit)	V12883	Cambridge Bioscience UK
Chloroform	C2432	Sigma Aldrich UK
Collagen Type I	C8919	Sigma Aldrich UK
Collagenase	C2674	Sigma Aldrich UK
Complete Freunds Adjuvant	F5881	Sigma Aldrich UK
Concanavalin A	C7275	Sigma Aldrich UK
Cytofix/Cytoperm Kit	554772	BD Bioscience UK
FasL PCR primers	N/A	Sigma Genosys, UK
Fluorescein (20%)	N/A	Moorfields Eye Hospital
Geneticin, G418	10131-019	Invitrogen UK
Gentamicin	G1397	Sigma Aldrich UK
Glutamine	25030-024	Invitrogen UK
Hanks balanced salts solution HBSS	24020-091	Invitrogen UK
HBSS without Ca^{2+} and Mg^{2+}	14170-088	Invitrogen UK
Heat inactivated Fetal Calf Serum	4-101-500	LabTech International UK
IL-2, recombinant, mouse	212-12	Peprotech UK
IL-2, recombinant, rat	PRP09BZ	Serotec UK
Infinity Cholesterol Assay Kit	TR13421	ThermoTrace Australia
IRBP Peptide	N/A	A.B.C. UK
Live-dead viability assay kit	L-3224	Cambridge Bioscience UK
Loading buffer blue/orange 6x	G1881	Promega UK
Lovastatin	438185	Calbiochem UK
Lympholyte Rat	CL5045	VH Bio UK
Methyl-^{3}H thymidine [25Ci/mMol]	TRK 300	Amersham Bioscience UK
Methyl α-D-mannopyranoside	M6882	Sigma Aldrich UK
Mevalonolactone	M4667	Sigma Aldrich UK

Mineral oil	M5904	Sigma Aldrich UK
MOG peptide	N/A	A.B.C. UK
Mouse Fas ligand ELISA	MFL00	R&D systems UK
Mouse IFN-γ ELISA	MIF00	R&D systems UK
Mouse IL-2 ELISA	M2000	R&D systems UK
Mouse IL-4 ELISA	M4000	R&D systems UK
Mouse IL-5 ELISA	M5000	R&D systems UK
Mouse IL-10 ELISA	M1000	R&D systems, UK
Mouse IL-12 p70 ELISA	M1270	R&D systems, UK
Mouse TGF-β1 ELISA	MB100	R&D systems, UK
Mouse TH1/TH2 CBA kit	551287	BD Bioscience UK
Non essential amino acids	11140-035	Invitrogen UK
Nutrient mix F10 Ham	31550-023	Invitrogen UK
Penicillin and Streptomycin Solution	15070-063	Invitrogen UK
Perm/Wash Buffer	554723	Pharmingen BD UK
Phosphate buffered saline	P4417	Sigma Aldrich UK
RNeasy Extraction kit	74104	Qiagen Ltd UK
RPMI 1640 with Glutamax	72400-021	Invitrogen UK
RPMI-1640 Dutch Modification	22409-015	Invitrogen UK
RPMI-1640 without L-Glutamine	31870-025	Invitrogen UK
SAg peptide	N/A	A.B.C. UK
Sodium pyruvate	11360-039	Invitrogen UK
Squalene	S3626	Sigma UK
SuperScript Kit	11904-018	Invitrogen UK
Taq DNA polymerase	600 132-81	Stratagene UK
Transwells 4μm Collagen Coated	3496	Corning BV UK
Transwells 4μm Tissue culture treated	3472	Corning BV UK
Trypsin EDTA solution	25300-062	Invitrogen UK
YoPro-1	Y3603	Cambridge Bioscience UK
β-actin PCR primers	N/A	Sigma Genosys UK

2.2 Antibodies

Antibody	Clone	Host	Isotype	Source
Anti-rat CD5-RPE	OX-19	Mouse	IgG1	Serotec
Anti-rat CD4-RPE	W3/25	Mouse	IgG1	Serotec
Anti-rat CD8 RPE-Cy5	OX-8	Mouse	IgG1	Serotec
Anti-rat CD25-RPE	OX-39	Mouse	IgG1	Serotec
Anti-mouse CD4-PerCP	RM4-5	Rat	IgG2a	BD Bioscience
Anti-mouse CD25-PE	PC615.3	Rat	IgG1	Caltag
Anti-mouse CD62L	MEL-14	Rat	IgG2a	Caltag
Anti-mouse IL-4-PE	11B11	Rat	IgG1	BD Bioscience
Anti-mouse IFN-γ-FITC	XMG1.2	Rat	IgG1	BD Bioscience
Isotype control PerCP	R35-95	Rat	IgG2a	BD Bioscience
Isotype control FITC	R3-34	Rat	IgG1	BD Bioscience
Isotype control PE	R3-34	Rat	IgG1	BD Bioscience
Isotype control RPE	F8-11-13	Mouse	IgG1	Serotec
Isotype control RPE-Cy5	MCA1209C	Mouse	IgG1	Serotec

Table 2.1 Directly conjugated primary antibodies used in these studies.

Antibody	Clone	Species	Isotype	Source
Anti-rat CD45	OX-22	Mouse	IgG1	Serotec
Anti-human CD95	DX2	Mouse	IgG1	BD Bioscience
Anti-human FasL	4H9	Hamster	IgG	Beckman Coulter
Anti-human FasL	4A5	Hamster	IgG	Beckman Coulter
Anti-rat FasL (biotinylated)	MFL4	Hamster	IgG3	BD Bioscience
Anti-human FasL	FSLO1	Mouse	IgM	Biocarta
Anti-rat ICAM-1	1A29	Mouse	IgG1	Serotec
Anti-mouse MHC class II	M5/114.15.2	Rat	IgG2b	e-bioscience

Table 2.2 Unconjugated primary antibodies used in these studies.

Secondary Reagents	Host	Specificity	Source
Streptavidin-PE		Biotin	BD Bioscience
Anti-mouse RPE (rat preabsorbed)	Goat	Mouse IgG	Serotec
Anti-hamster FITC	Goat	Mouse IgG	Serotec
Anti-mouse FITC	Donkey	Mouse IgG H+L chain	Jackson Laboratories
Anti-mouse FITC	Rabbit	Mouse IgM μ chain specific	Rockland
Protein A/G-Horse Radish Peroxidase		Mouse	Pierce
Streptavidin Texas Red		Biotin	Amersham Bioscience

Table 2.3 Secondary antibodies and reagents used in these studies.

2.3 Animals

Lewis rats, B10.RIII^{7INS} mice and C57BL/6 mice were purchased from Harlan Olac (Bicester, UK). All animal studies were conducted in accordance with the institutional guidelines and UK Home Office regulations. All animals were provided with RM1(E) feed and water *ad libitum* and terminated using a schedule 1 method of killing.

2.4 Stock Solutions

A number of solutions were prepared, aliquoted and stored as described as follows. This was in order to prevent degradation of the reagents as a result of multiple freeze thaw cycles and also to prevent contamination of stock solutions.

2.4.1 2-Mercaptoethanol

2-mercaptoethanol (2ME; molecular weight 78.13) was purchased as a 14.3M stock solution. 14.3M 2-ME was diluted in phosphate buffered saline [1/14.3 v/v] (PBS; see section 2.4.17). In aseptic conditions this was then sterilised by passing through a 0.22μM filter and aliquoted in 500μl volumes and stored at -20°C until use. At this time 2-ME was thawed and further diluted [1/50000 v/v] in culture medium to give a final concentration of $2x10^{-5}$M.

2.4.2 C3-transferase

C3-transferase (C3 exoenzyme from *Clostridium Botulinum*) was prepared as described by Adamson *et al* 1999.

2.4.3 Calcein AM

A working concentration of Calcein AM [0.2μM] was prepared by a 1/20000 dilution in PBS (see section 2.4.17).

2.4.4 Collagen Solution Type I

In aseptic conditions Type I collagen solution was diluted 1 in 20 (v/v) in HBSS immediately prior to use in collagen coating of tissue culture wares.

2.4.5 Collagenase

In aseptic conditions, collagenase was resuspended at 1mg/ml in sterile calcium and magnesium free HBSS. Collagenase was then aliquoted in 1ml volumes and stored at -20°C until required for use. Aliquots were then thawed and used immediately.

2.4.6 Concanavalin A (Con A)

In aseptic conditions lyophilised Concanavalin A was resuspended at 1mg/ml in sterile RPMI-1640. Con A was then aliquoted in 1ml volumes and at stored at -20°C until required for use. Aliquots were then thawed and stored at 4°C for up to 3 days.

2.4.7 CNS EC medium

In aseptic conditions 100mls of media was removed from a 500ml bottle of Nutrient mix Ham's F-10. The media was then supplemented with FCS [20% v/v] and penicillin and streptomycin solution [100U/ml and 100μg/ml].

2.4.8 Ethidium Homodimer-1

A working concentration of Ethidium Homodimer-1 (EthD1 [0.1μM]) was prepared by a 1/20000 dilution in PBS (see section 2.4.17).

2.4.9 FCS

In aseptic conditions FCS was thawed in a 37°C water bath. After complete thawing FCS was either heat-inactivated at 56°C in a water bath for 45 min or if purchased already heat-inactivated, aliquoted in 50ml volumes. FCS was then stored at -20°C until required and thawed in a 37°C water bath when required for tissue culture purposes.

2.4.10 Glutamine Solution

Glutamine solution [200mM] was thawed in a 37°C water bath and aliquoted in 5ml volumes. Aliquots were then refrozen and thawed as required in a 37°C water bath for tissue culture media. Glutamine was then further diluted in tissue culture media.

2.4.11 Lovastatin

Lovastatin was brought into suspension by dissolving in dimethyl sulfoxide (DMSO; 100%). This solution was aliquoted, stored at -20°C and thawed as required.

2.4.12 Methyl ^{3}H-thymidine

In aseptic conditions 1mCi/ml methyl ^{3}H-thymidine was diluted 1 in 10 in RPMI-1640 to give a 100μCi/ml solution. 10μl or 5μl of diluted methyl ^{3}H-thymidine was added as the equivalent of 1μCi or 0.5μCi per 200μl/well of a 96-well assay plate, respectively.

2.4.13 Methyl-α-D-mannopyranoside

A 1M solution of methyl-α-D-mannopyranoside (molecular weight 194.2) was prepared by dissolving 1.942g methyl-α-D-mannopyranoside in 10ml of RPMI 1640. This solution was prepared immediately prior to use and not stored.

2.4.14 Non CNS EC medium

In aseptic conditions 50ml of media was removed from a 500ml bottle of RPMI-1640. The bottle was then supplemented with FCS [10% v/v], L-glutamine [2mM] and Penicillin and streptomycin solution [100U/ml and 100μg/ml].

2.4.15 Non-essential amino acids (NEAA) Solution

Sterile NEAA solution [100x stock] was aliquoted into 5ml volumes. Aliquots were then stored at 4°C and used as required.

2.4.16 Normal Rat Serum (NRS)

Normal rat serum was obtained by cardiac puncture of specifc pathogen-free rats. Whole blood was stored in sodium heparin coated vacutainers at 4°C overnight. This was then centrifuged at 260g for 10 min, the serum removed and aliquoted into 2ml volumes. NRS was heat-inactivated by incubation in a 56°C water bath for 45 min. The serum was then stored at -20°C until required. At this time NRS was thawed in a 37°C water bath and used for tissue culture media.

2.4.17 PBS

PBS was prepared following instructions provided by the manufacturer. Briefly one PBS tablet was dissolved in 200ml of 18Ω water on a magnetic stirrer. Sterile PBS was prepared in aseptic conditions by sterilisation through a 0.22μM filter using a 25ml syringe.

2.4.18 Penicillin and Streptomycin Solution

Sterile penicillin and streptomycin solution [10000U/ml and 10000μg/ml] was thawed in a 37°C water bath. The solution was then aliquoted into 10ml volumes and re-frozen at -20°C until required. Aliquots were thawed as required for tissue culture media.

2.4.19 Permwash Buffer

Concentrated Permwash buffer was diluted 1in 10 in 18Ω water. This solution was used or stored for up to 7 days at 4°C.

2.4.20 Propidium Iodide

Propidium Iodide (PI) was resuspended at 1mg/ml in 18Ω water and aliquoted in 100μl volumes into 500μl microfuge tubes. Aliquots were then stored at -20°C and thawed as required. Once thawed, PI solution was stored at 4°C.

2.4.21 PLN T cell medium

This medium was prepared for PLN T cells to be used for the purpose of adhesion assays. In aseptic conditions 65ml of media was removed from a 500ml bottle of RPMI 1640 with L-alanyl-L-glutamine. The bottle of media was then supplemented with FCS [10% v/v], penicillin and streptomycin solution [100units and 100μg/ml], sodium pyruvate solution [1mM], NEAA [1mM] and 2-ME [$2x10^{-5}$]. This was then stored at 4°C until use.

2.4.22 Rat IL-2 (IL-2-containing supernatant)

Supernatant was harvested from the mouse IL-2 transfected C127 mammary tumour cell line and sterilised through a 0.22μM filter under aseptic conditions. Supernatant was then aliquoted into 5ml volumes and stored at -20°C until required for assay or culture at which time it was thawed in a 37°C water bath and subsequently stored at 4°C for up to 7days. However this will further be referred to as rat IL-2.

2.4.23 Serotec Rat IL-2

Rat recombinant IL-2 [5000U/ml] (Serotec) was aliquoted into 50μl volumes and stored at -20°C. When required, aliquots were thawed and used by making a further dilution [1/50 v/v] in culture medium.

2.4.24 Sodium Pyruvate (SP) Solution

Sterile SP solution was aliquoted into 5ml volumes. Aliquots were then stored at 4°C and used as required.

2.4.25 Squalene

In aseptic conditions squalene was made into a 5mg/ml solution in ethanol.

2.4.26 T cell Medium

The following T cell medium was prepared for the culture of rat and mouse splenocyte and PLN T cells for the establishment of T cell lines, T cell cytokine production or T cell proliferation. In aseptic conditions 65ml of media was removed

from a 500ml bottle of RPMI 1640 buffered with 25mM HEPES and 1g/L sodium bicarbonate. The media was then supplemented with FCS [10% v/v], L-Glutamine [2mM], gentamicin [50μg/ml], SP [1mM], NEAA [1mM] and 2-ME [$2x10^{-5}$ M] and stored at 4°C.

2.4.27 T cell medium for antigenic restimulation

In aseptic conditions a 500ml bottle of RPMI- 1640 buffered with 25mM HEPES and 1g/L sodium bicarbonate was supplemented with L-Glutamine [2mM] and gentamicin [50μg/ml]. 194mls of this was then transferred to a sterile tissue culture bottle and further supplemented with NRS [1% v/v], SP [1mM], NEAA [1mM] and 2-ME [$2x10^{-5}$ M]. This was then stored at 4°C until use.

2.4.28 Wash Buffer

In aseptic conditions 25ml of media was removed from a 500ml bottle of RPMI-1640 buffered with 25mM HEPES and 1g/L sodium bicarbonate. This was then supplemented with FCS [5% v/v] and L-Glutamine [2mM].

2.4.29 YoPro-1

A working dilution of YoPro-1 [0.5μM] was prepared by a 1/2000 dilution in PBS (see section 2.4.17)

2.5 Tissue Culture

2.5.1 Thawing of Cells

Cells cultured from frozen stocks were defrosted quickly at 37°C in a water bath and washed in 10mls of respective warmed medium and centrifuged at 260g for 10 min in an MSE Harrier 18/80 centrifuge (Sanyo Gallenkamp PLC, UK). Cells were counted in a haemocytometer and viable cells, as determined by Trypan Blue exclusion, were set up at $2x10^5$ cells/ml in respective media. After washing, endothelial cells were transferred directly to a T75cm^2 vented tissue culture flask in the respective tissue

culture media. All cells were then cultured at 37°C, 5% CO_2 humidified incubator (Phillip Harris, UK).

2.5.2 Freezing of cells

Cells were set up at a known density in the respective media and 900µl volumes were transferred to 1.8ml cryotubes. Cells were then incubated on ice for 10 min and supplemented with DMSO [10% v/v] added dropwise. Cryotubes were then wrapped in tissue and transferred to a polystyrene box within a -70°C freezer for up to 72h before transfer to liquid nitrogen (LN_2) for long term storage.

2.5.3 Isolation of a single cell suspension

Single cell suspensions of rat splenic, cervical and mesenteric lymph node T cells and thymocytes were prepared from tissues isolated from 4-8 week old female Lewis rats. Single cells suspensions of splenic T cells and PLN T cells were also prepared following this protocol from Lewis Rats, B10.RIII^{INS} and C57BL/6 mice at expected peak of disease. Cell suspensions were prepared by mechanical disruption of the tissue and passage through a 0.75µm sterile sieve using the plunger of a 5ml syringe. Cells were washed twice with 50ml of wash buffer and centrifugation at 260g for 10 min. Lymphocytes were isolated and stored in wash buffer (see section 2.4.28) on ice until processing.

2.6 T lymphocytes

2.6.1 Thymocytes

Thymocytes were prepared (as described in section 2.5.3) and resuspended in a known volume of T cell medium. Cells were then irradiated with 2000rads on a Caesium^{137} source (GammaCell Irradiator, MDS Nordion, courtesy of the London School of Hygiene and Tropical Medicine, London UK). Irradiated cells were then added to T cells in culture at a 50:1 ratio of feeder cells to T cells (Sedgewick *et al* 1989). Three days later dead feeder cells were removed from the culture by density

centrifugation of cultures on Lympholyte Rat at 720g for 20 min (Figure 2.1). In this process dead cells precipitate at the bottom of the centrifuge tube whilst the live T cells form a layer above the Lympholyte.

2.6.2 IL-2 transfected C127 mammary tumor cells.

A mouse mammary tumour cell line transfected to constitutively secrete mouse IL-2 kindly donated by Dr F. Melchers, Bern (Karasuyama and Melchers 1988), was cultured at a density of $2x10^5$ cells/ml in T cell medium (see section 2.4.26). Cells were provided with fresh medium every 3-4 days. These hybridoma cells were G418 resistant and regularly, IL-2 secreting cells were selected by culturing in the presence of Geneticin (G418) [1mg/ml] then cultured for 3-4 days in the absence of G418 prior to culture at $4x10^5$ cells/ml for 2-3 days, after which time the supernatant was harvested. The supernatant was then sterile-filtered and stored at -20°C until required for assay or T cell culture. All batches of IL-2 containing supernatant were tested for their ability to stimulate T cell proliferation using an IL-2 dependent T cell line and the optimum concentration determined (see section 2.8.1). This source of IL-2 is further referred to as rat IL-2. Cell viability and growth was monitored using a haemocytometer and Trypan blue exclusion.

2.6.3 Mitogen specific T cell lines

Lewis rat splenocytes prepared as described (section 2.5.3) were then set up at $1x10^6$cells/ml in T cell medium (see section 2.4.26) and stimulated with Con A [5μg/ml] (Zhao *et al* 1994). Cells were re-stimulated with autologous Lewis thymocytes (see section 2.6.1) and Con A [5μg/ml] every 7-10 days and maintained between re-stimulation every 3-4 days with rat IL-2 (see section 2.4.22). T cells were cultured and re-stimulated with mitogen and feeders for at least 3-4 passages, thus establishing a Con A driven T cell line prior to use in the subsequent experiments. Cell viability and growth was as described previously (see section 2.6.2).

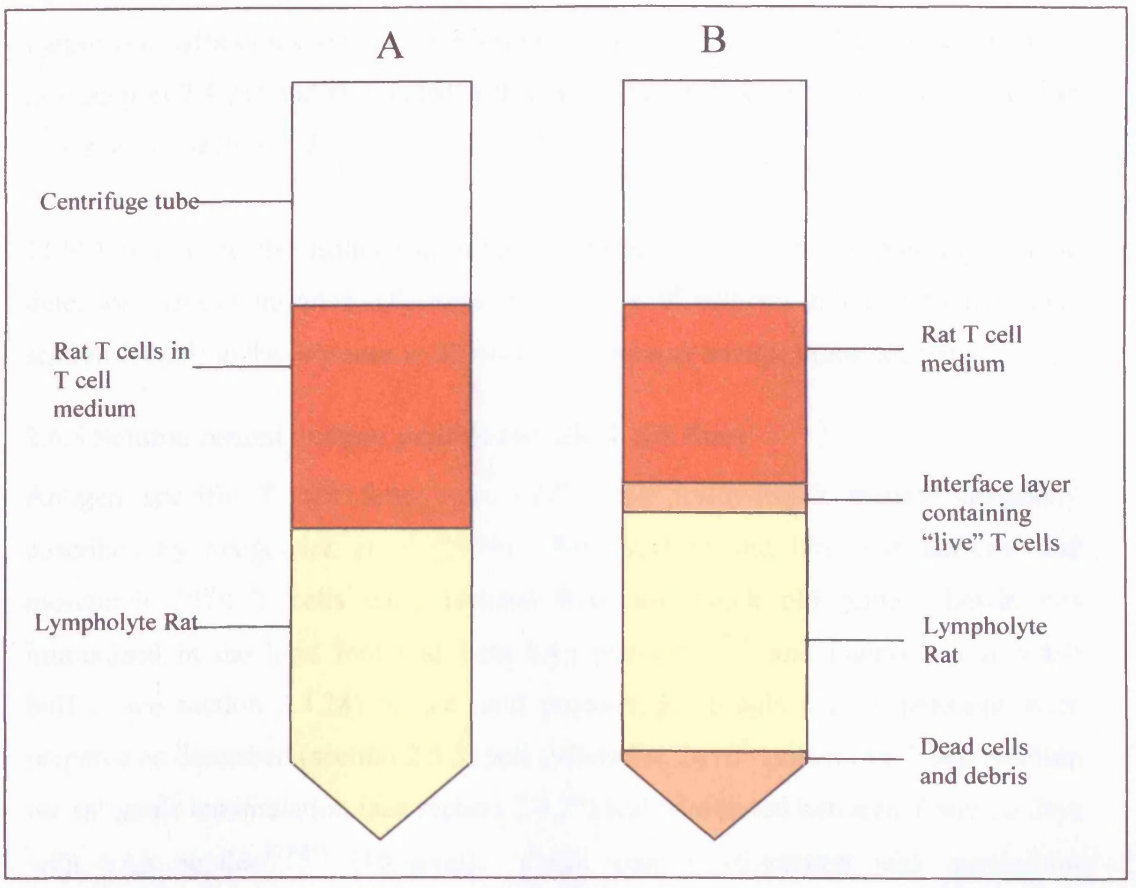

Figure 2.1 The principles of density centrifugation.
Tube A shows the layering of the cell suspension to the Lympholyte Rat prior to centrifugation. Tube B shows the separation of the cells post centrifugation. The live cells are prevented from passage through the Lympholyte due to size exclusion and thus form an "interface layer or buffy coat" on top of the Lympholyte rat. Dead cells and debris pass freely through the density gradient and sediment at the bottom of the tube. The interface layer containing the live cells is then carefully aspirated and the cells washed thoroughly to remove any traces of Lympholyte.

2.6.4 PLN T cells

PLN T cells were isolated and prepared as described in section 2.5.3. Cells for the purposes of adhesion assays were cultured at $1x10^6$ cells/ml in rat PLN T cell medium (see section 2.4.21) and stimulated with Con A [5μg/ml] for at least 24h prior to use in assay (see section 2.8.6).

PLN T cells were also isolated from EAU and EAE mice for the purpose of cytokine detection. In this instance cells were set up at $2x10^6$ cells/ml in T cell medium (see section 2.4.26) in the presence or absence of antigen or mitogen (see section 2.13.19).

2.6.5 Soluble retinal antigen peptide specific T cell lines

Antigen specific T cell lines were established following a method originally described by Sedgewick *et al* (1989). Briefly, fore and hind leg, cervical and mesenteric PLN T cells were isolated from 4-6 week old female Lewis rats immunized in the hind foot pad with SAg peptide$^{273-289}$ and maintained in wash buffer (see section 2.4.28) on ice until processing. Single cell suspensions were prepared as described (section 2.5.3) and cultured at $2x10^6$ cells/ml in T cell medium for antigenic restimulation (see section 2.4.27) and stimulated between 7 and 10 days with SAg peptide$^{273-289}$ [10μg/ml]. Cells were re-stimulated with autologous thymocytes (see section 2.6.1) and SAg peptide$^{273-289}$ [10μg/ml] every 7-10 days and maintained between re-stimulation every 3-4 days with rat T cell medium (see section 2.4.26) containing rat IL-2 (see section 2.4.22). T cells were cultured and re-stimulated with antigen and feeders for 3-4 passages establishing a SAg peptide specific T cell line prior to use in the subsequent experiments. All migration studies (see section 2.8.2, 2.8.3, 2.8.8) were performed using T cells 4-5 days post-antigenic restimulation (3 days with peptide and feeder APC, 1-2 days post IL-2), whilst at their migratory peak (Pryce *et al* 1997). Cell viability and growth was monitored as described previously (see section 2.6.2).

2.6.6 HT-2 Cell line

An IL-2 dependent murine helper T cell line HT-2 (Watson 1979) was cultured in order to assay for IL-2 activity in the C127 cell supernatants. Cells were cultured at a density of $2x10^5$ cells/ml in T cell medium (see section 2.4.26) in the presence of rat IL-2 (see section 2.4.22). Fresh medium was added every 3-4 days until required for IL-2 bioassay. Cell viability was determined as described for T lymphocytes.

2.6.7 L1210 cells

Parental L1210 ($L1210^{lo}$, $L1210^-$) and human Fas-Fc transfected L1210 ($L1210^{hi}$, $L1210^+$, L 1210Fas) mouse lymphocytic leukaemic cell lines (Rouvier *et al* 1993) were cultured *in vitro* at a density of $2x10^5$ cells/ml in T cell medium (see section 2.4.26). Cells were provided with fresh medium every 3-4 days until required for functional Fas ligand assay. Cell viability was again determined as for T lymphocytes. These cells will further be referred to as L1210 and L1210Fas cells.

2.7 Endothelial Cells

2.7.1 Cell Dissociation

In preparation for dissociation of EC monolayers, culture medium was aspirated and the monolayers washed three times with calcium-and-magnesium-free (Ca^{2+} and Mg^{2+} free) HBSS to remove traces of serum and Ca^{2+} and Mg^{2+} essential for cell attachment. For routine tissue culture purposes, monolayers were then incubated with Trypsin/EDTA [1X] for 5 min at 37°C, 5% CO_2. For cell dissociation prior to cell surface staining of EC for flow cytometry, monolayers were dissociated with collagenase [1mg/ml] at 37°C 5%CO_2 until cells had detached. Detachment was confirmed by phase-contrast light microscopy and cells were collected into 10mls of their respective serum-containing medium. Cells were then centrifuged at 260g for 10 min prior to replating or distribution into 1.5ml microfuge tubes required for staining purposes.

2.7.2 Collagen Coating

Tissue culture plastics required for the culture of CNS derived EC (e.g. GP8/3.9 and JG2.1, LD7.4) were coated with a type I collagen (see section 2.4.4) for 1h at room temperature (RT). The solution was then aspirated and the collagen cross-linked by placing the flask/dish in an ammonia hydroxide vapour chamber for 20 min. Conversion of the collagen solution from yellow to pink was confirmed and the collagen-coated surface of the flask/dish was subsequently washed with HBSS until the phenol red of the HBSS remained red, indicating a neutral pH.

2.7.3 Central Nervous System-derived Endothelial Cells (CNS EC)

2.7.3.1 Brain microvascular EC

The SV40 "large T" immortalised Lewis rat brain endothelial cell line, GP8/3.9, representing the BBB was cultured *in vitro* on type I collagen-coated tissue culture flasks (Greenwood *et al* 1996). Cells were cultured in CNS EC medium (see section 2.4.7), which was changed every 3-4 days until the formation of confluent monolayers (3-6 days). Cells were plated onto collagen-coated tissue culture plastic at a maximum split ratio of 1 in 5. All experiments were performed on EC monolayers between passages 15 and 20.

2.7.3.2 Retinal microvascular EC

The SV40 "large T" immortalised Lewis rat retinal endothelial cell line, JG2.1 representing the vascular BRB was cultured *in vitro* on Type I collagen-coated tissue culture flasks (Greenwood *et al* 1996). Cells were cultured in CNS EC medium (see section 2.4.7). Culture medium was changed every 3-4 days until the formation of confluent monolayers (3-6 days). Cells were plated onto collagen-coated tissue culture plastic at a maximum split ratio of 1 in 5. All experiments were performed on EC monolayers between passages 15 and 20.

2.7.3.3 Retinal pigment epithelial cells

The SV40 "large T" immortalised PVG ($RT1^C$) rat retinal pigment epithelial cell line, LD7.4 representing the posterior BRB was cultured *in vitro* in CNS EC medium (see section 2.4.7; Greenwood *et al* 1996). Culture medium was changed every 3-4 days until the formation of confluent monolayers (3-6 days). Cells were plated directly into tissue culture flasks at a maximum split ratio of 1 in 5. All experiments were carried out on cells between passages 19 and 23.

2.7.4 Non-Central Nervous System-derived Endothelial Cells.

2.7.4.1 HEV

The high endothelial venule (HEV) endothelial cell line, a kind gift from A. Ager (NIMR, Mill Hill) was cultured in non-CNS EC medium (see section 2.4.14). Culture medium was again changed every 3-4 days until the formation of confluent monolayers. Cells were plated directly into tissue culture wares at a maximum split ratio of 1 in 5. All experiments were carried out on cells between passages 15 and 20.

2.8 Cellular Assays

2.8.1 IL-2 Bioassay

IL-2-containing supernatants were assayed for their ability to support T cell proliferation by means of the IL-2 bioassay (Zhao *et al* 1994).

Prior to setting up an IL-2 bioassay, the appropriate number of IL-2 dependent HT-2 cells were washed twice in T cell medium (see section 2.4.26) by centrifugation at 260g for 10 min in order to remove residual IL-2 that may be present within the culture. $2x10^4$ HT-2 cells in a volume of 100µl of T cell medium were added to 39 wells of a flat-bottomed 96 well plate per IL-2-containing sample to be assayed. HT-2 cells were then incubated in triplicate with no IL-2, or increasing concentrations of

IL-2 supernatant [0-15%] (see Figure 2.2). Commercially available recombinant rat IL-2 (recombinant rat IL-2, see section 2.4.23) or a previously assayed rat IL-2 (see section 2.4.22) with known optimal activity was set up as a standard positive control. Each well was made up to a final volume of 200µl with T cell medium. Cells were cultured for 2-4 days at 37°C and 5% CO_2 until HT-2 cells cultured in the absence of IL-2 appeared as resting non-proliferating cells at which time they were then pulsed with 0.5µCi overnight or 1µCi of methyl ^{3}H-thymidine for 8 hours. Cells were adsorbed onto nitrocellulose filters using a Dynatech Cell harvester. The uptake of radiolabel by proliferating cells was measured by β scintillation counting using Packard Ultima Gold F scintillation fluid and a Canberra Packard 1600TR liquid scintillation analyser.

As illustrated in Figure 2.2 the optimum concentration of IL-2 for each supernatant was determined by the lowest concentration required to support maximal proliferation.

2.8.2 Non-contact EC assay

$1x10^4$ BEC and HEV EC were plated into collagen-coated and untreated wells of a 24 well plate respectively. At confluence EC culture medium was aspirated and monolayers washed three times with 1ml HBSS. The media was then replaced with 600µl of T cell medium (see section 2.4.26) containing rat IL-2 (see section 2.4.22). 2×10^5 syngeneic Con A-activated T cell line cells in 100µl of T cell medium containing IL-2 were added to 0.4µm Costar transwell inserts (which do not permit T cell migration) and incubated for 24, 48 or 72h either alone or in the presence of EC cultured in the wells beneath the insert (Figure 2.3). Control T cells were incubated in T cell medium containing IL-2 for the duration of the experiments to determine basal levels of apoptosis, to prevent IL-2 deprivation-induced apoptosis (Akbar *et al* 1993). T cells were harvested from the transwell insert by washing twice with 100µl of PBS. Cells were then stained for flow cytometric analysis as described in section 2.9.2.

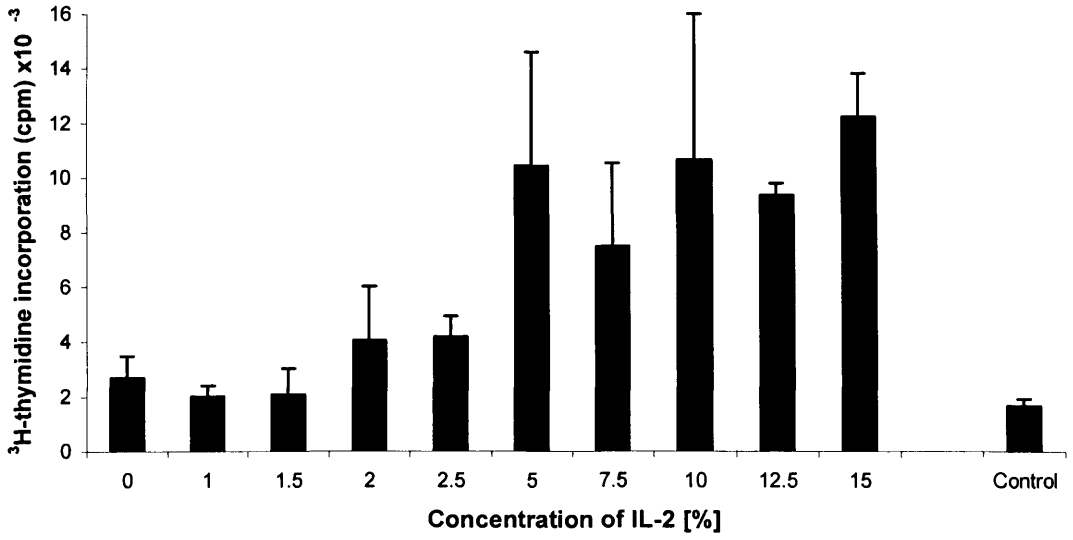

Figure 2.2 Proliferation of HT2 cells as quantitated by methyl ^{3}H-thymidine incorporation in response to increasing concentrations of rat IL-2.
The optimum concentration of IL-2 was determined as the lowest concentration to support maximal proliferation. In this instance the optimum final concentration of IL-2 was determined as 5% since at 5% IL-2, cells were observed to proliferate at levels comparable to that of 10% and 15% IL-2. Data is shown as the means of triplicate wells ± S.D.

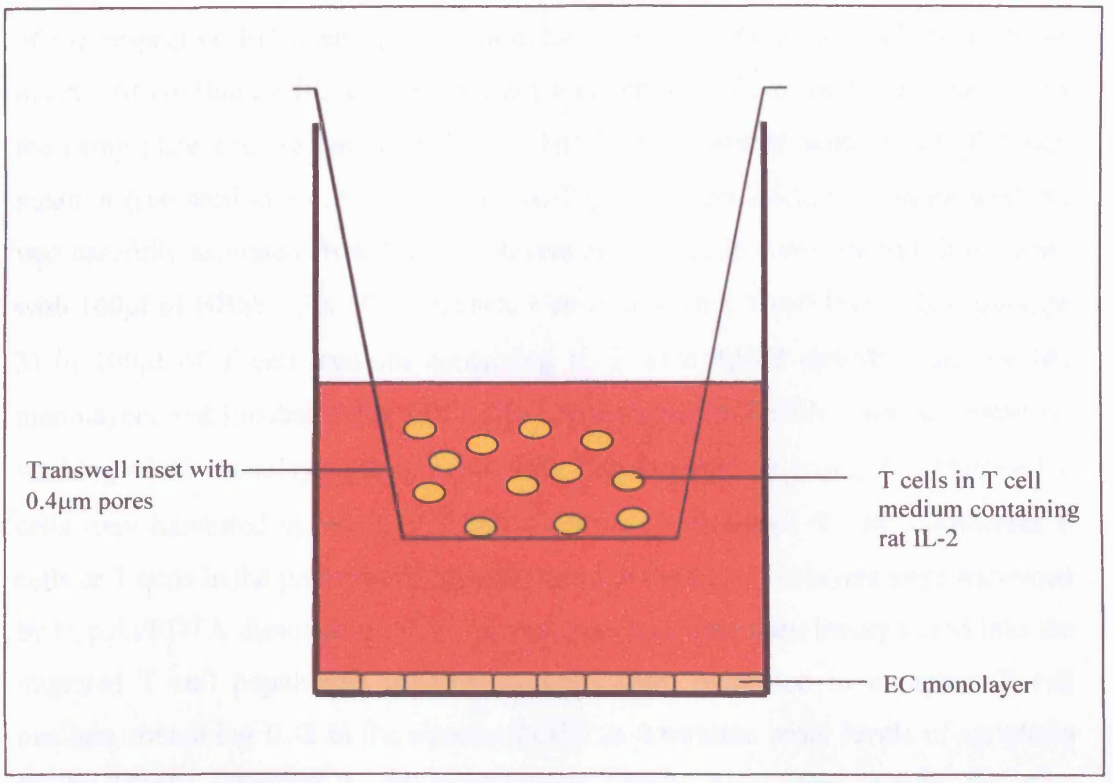

Figure 2.3 showing the relationship of T cells to EC in the non-contact assay.
T cells are cultured for 24, 48 or 72h within inserts, above EC monolayers cultured in the wells beneath. During assay all cells were cultured in T cell medium containing rat IL-2. The 0.4μm inserts prevent contact of T cells with EC.

2.8.3 Transmigration Assay

$1x10^4$ BEC and HEV EC were plated out into 3.0μm collagen-coated or tissue culture treated Costar transwell inserts respectively, and cultured in 24-well plates with 600μl of the respective EC media (see section 2.4.7 and 2.4.14) in the well beneath the insert. At confluence EC culture medium was aspirated from the lower chamber of the assay plate and washed with 1ml of HBSS and replaced with 600μl of T cell medium (see section 2.4.26) containing IL-2 (see section 2.4.22). Culture medium was carefully aspirated from the monolayers and the cells were washed three times with 100μl of HBSS. 3×10^5 syngeneic Con A-activated T cell line cells (>passage 3) in 100μl of T cell medium containing IL-2 were added directly onto the EC monolayers and incubated for 24 or 48h. Non-migrated T cells were harvested by washing of the monolayers three times with 100μl of PBS (Figure 2.4). Migrated T cells were harvested by washing of the lower well with 600μl of PBS. Adherent T cells or T cells in the process of migrating through the EC monolayers were harvested by trypsin/EDTA dissociation of the monolayers and were then incorporated into the migrated T cell population. Control T cells were incubated in complete T cell medium containing IL-2 in the absence of EC to determine basal levels of apoptosis in the highly activated T cell populations. Cells were then stained for flow cytometric analysis as described in section 2.9.2.

2.8.4 Trucount Assay

The levels of cells which migrated through BEC and HEV monolayers were quantitated using the BD Trucount assay. The assay was performed according to the manufacturer's instructions. Briefly migrated T cell populations (see Figure 2.4) were recovered from the lower chamber and incubated in BD Trucount tubes. Cells were then stained with anti rat-CD5-PE and then aquired on a BD FACScan cytometer.

Absolute cell numbers were determined by the number of $CD5^+$ cells divided by the number of beads multiplied by the bead concentration.

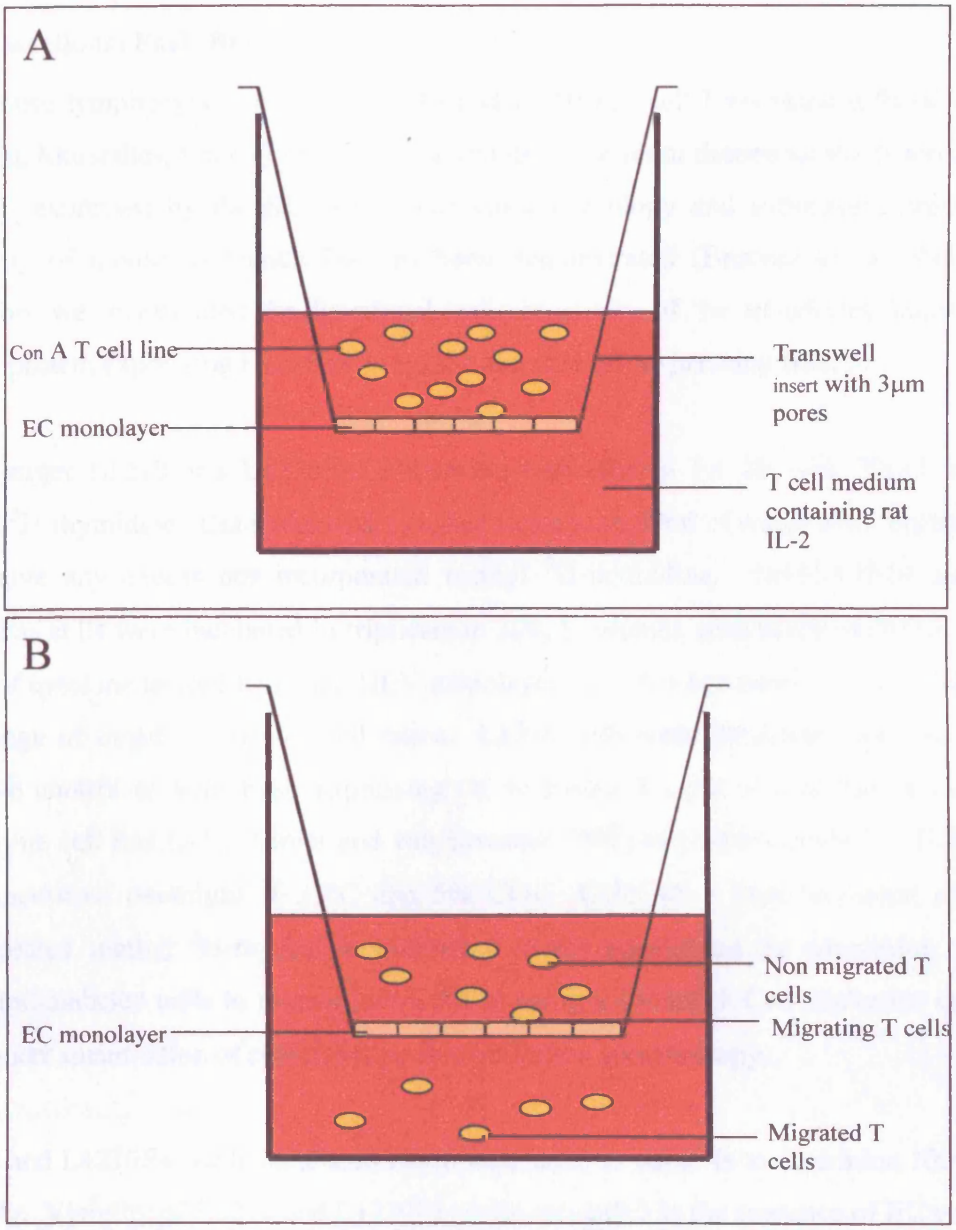

Figure 2.4 showing the relationship between T cells and EC during the transmigration assay.

Panel A; EC monolayers were cultured within the insert and T cells added for 24, 48 or 72h directly to confluent monolayers. During assay all cells were cultured in rat T cell medium in the presence of rat IL-2. The transwell inserts contain 3.0μm pores, which permit only active migration of T cells to the lower chamber. Panel B shows the T cell populations investigated in the transmigration assay. Non-adherent or loosely adherent T cells within the insert were harvested and comprised the "non-migrated" T cell population. T cells in the well beneath were harvested and comprised the "migrated" T cell population. Firmly adherent T cells or those in the process of migration are obtained by trypsinisation of the EC monolayer and incorporated into the migrated T cell population.

2.8.5 Functional FasL Bioassay

The mouse lymphocytic leukaemic L1210 and L1210Fas cell lines (kind gifts of P. Golstein, Marseilles, France) were cultured *in vitro* in order to determine the function of FasL expressed by the EC lines. The close homology and subsequent cross-reactivity of mouse to human Fas has been demonstrated (Brunner *et al* 1995). Therefore we investigated the functional cross reactivity of the transfected human Fas-Fc protein expressing L1210 cells against the rat FasL expressing BEC.

$2x10^6$ target L1210 and L1210Fas cells were radiolabeled for 2h with 20μCi of methyl ^{3}H-thymidine. Cells were then washed twice with 50ml of warm wash buffer to remove any excess non-incorporated methyl ^{3}H-thymidine. $1x10^5$ L1210 and L1210Fas cells were incubated in triplicate in 100μl volumes with unstimulated or a range of cytokine treated BEC and HEV monolayers in a flat-bottomed 96 well plate at a range of target to effector cell ratios. L1210 cells were incubated alone as a negative control or with FasL expressing rat or mouse T cells or a human B cell lymphoma cell line (J-Y; Palmer and van Seventer 1997) as positive controls. Cells were incubated overnight at 37°C and 5% CO_2. Cells were then harvested and incorporated methyl ^{3}H-thymidine radiolabel levels quantitated by adsorption of intact radiolabeled cells to nitrocellulose filters using a Dynatech Cell Harvester and subsequent quantitation of radiolabel by β-scintillation spectroscopy.

L1210 and L1210Fas cells incubated alone were used as controls to determine 100% viability. Viability of L1210 and L1210Fas cells incubated in the presence of EC was expressed as a percentage of control viability. FasL mediated cytotoxicity was calculated as the loss of cell viability represented by a loss of radiolabeled L1210Fas cells using the following equation:

$$\frac{(\text{cpm of control labelled cells}) - (\text{cpm of labelled cells} + \text{EC})}{\text{cpm of control labelled cells}} \times 100$$

2.8.6 T cell viability

The viability and cell surface expression of the rat leukocyte surface antigen CD5 and ICAM-1 on T-lymphocytes and retinal EC respectively, in response to lovastatin treatment was determined by PI staining and flow cytometry. Viability was determined by PI exclusion (Pavlik *et al* 1985).

Lewis rat Con A T cell line cells were set up at $1x10^6$ cells/ml in T cell medium (see section 2.4.26) containing IL-2 (see section 2.4.22) in the absence or increasing concentrations of lovastatin [0.1-100μM] and cultured for up to 5 days. $1x10^6$ T cells were harvested at each timepoint into 1.5ml microfuge tubes. Cells were centrifuged at 336g for 5 min and the supernatant carefully removed. Cells were then agitated and incubated with 4μl of anti-CD5-RPE for 30 min on ice in the dark. Cells were then washed with 1ml of PBS and centrifuged at 336g for 5 min. The cells were again agitated and resuspended in 200μl of PBS. Samples were then transferred to FACS tubes for acquisition on the flow cytometer. Immediately prior to the acquisition of each sample, 2 μl of PI [1mg/ml] was added to each sample tube and to the relevant controls. CD5 PE was detected within FL2 while PI emits at wavelength 617 nm that was detectable within the FL3 channel of the FACScan.

EC monolayers either untreated or pretreated for 24h with increasing concentrations of lovastatin [0.1-100μM] or C3 transferase [10μg/ml] were prepared for flow cytometric analysis as described in section 2.7.1. EC were then stained with 50μl/tube of the 1A29 anti-rat ICAM-1 antibody [10μg/ml]. Cells were incubated on ice for 30 min in the dark. Cells were then washed with 1ml of PBS and centrifuged at 336g for 4 min. The supernatant was poured off and the pellet agitated. Cells were then incubated with 50μl of donkey anti-mouse IgG-FITC [1/50 v/v] in PBS for 30 min on ice in the dark before washing with 1ml of PBS. Cells were centrifuged at 336g for 4 min, the supernatant was poured off and the pellet agitated. The cells were then resuspended in 200μl of PBS and transferred to FACS tubes for acquisition. Prior to acquisition 2 μl of PI [1mg/ml] was added to each sample tube and the

relevant controls. ICAM-1 FITC was detected within the FL1 channel while PI was detected in FL3.

For control purposes four samples of $1x10^6$ T-lymphocytes or EC were permeabilised using a Cytofix/Cytoperm kit. 100µl of cytofix/cytoperm was added to each sample tube and cells incubated on ice for 20 min in the dark. Cells were either unstained to determine the live population, single stained for CD5-PE, ICAM-1 FITC or PI and used to determine compensation and also to determine PI positive and negative cells or double stained for CD5 and PI or ICAM-1 and PI to confirm compensation settings. Cells were stained as for test samples however post permeabilisation with Cytofix/cytoperm, cells were washed with 1 ml of Perm/Wash. Control tubes were resuspended in 200µl of PBS and transferred to FACS tubes prior to acquisition. 2µl of PI [1mg/ml] was added immediately prior to acquisition to the relevant controls.

Cells were acquired using CellQuest software (BD). Gates were set to exclude non-viable cells on forward scatter and side scatter plots, counting at least 10000 events within the gate. PI stained permeabilised cells were used to determine live and dead cells. Cells exhibiting a mean fluorescence intensity (MFI) of $>10^3$ were classified as PI positive and thus dead. Data was expressed as a percentage of untreated viable cells.

2.8.7 PLN-Lymphocyte Adhesion Assay

PLN T cells were prepared as described previously (see sections 2.5.3, 2.6.4), activated with ConA [5µg/ml] for at least 24h prior to use in the following adhesion assay.

Con-A activated PLN cells were labelled with Calcein AM [1µM] at $5x10^6$ cells/ml in PLN T cell medium for 30 min at 37°C. Cells were then washed with wash buffer and $1x10^5$ fluorescently labelled cells in 100µl PLN cell medium (see section 2.4.21) were added to duplicate plates of untreated, statin, mevalonolactone or squalene pretreated REC or RPE monolayers and incubated for 90 min at 37°C, 5% CO_2. The

wells of one plate were then washed 6 times to remove non adherent cells with warmed Ca^{2+} and Mg^{2+} free HBSS and replaced with 100µl PLN cell medium. The unwashed plate was used to determine total adhesion. Plates were then centrifuged at 200g for 5 min at 20°C to collect cells at bottom of wells. Plates were then read on a Tecan Safire fluorescent plate reader taking an average of 10 readings of 9 points within each well. Readings were normalized by the subtraction of values for medium alone.

The percentage of cell adhesion was calculated as the ratio of adhesion mediated fluorescence (washed plate) as a percentage of total fluorescence per well (unwashed plate). Percentage adhesion for treatment groups was expressed as a percentage of control. Twelve wells were assayed per condition per experiment. Each experiment was performed in triplicate.

2.8.8 Time-lapse video microscopy transmigration assay

The effect of lovastatin treatment of EC upon T cell migration was investigated using a method of time-lapse video microscopy previously developed within the laboratory (Pryce *et al* 1997, Adamson *et al* 1999).

REC and RPE cells were cultured in flat-bottomed 96 well plates until confluent. At confluence, cells were either untreated or pretreated with increasing concentrations of lovastatin [0-100µM] in the presence or absence of mevalonolactone or squalene. EC monolayers were washed three times with 200µl of HBSS and, at 10 min intervals, $2x10^4$ SAg specific T cells in 100µl of rat T cell medium (see section 2.4.26) containing rat IL-2 (see section 2.4.22) were added to the EC monolayers. Cells were allowed to settle and migrate over a 4h period at 37°C, 5% CO_2. T cell migration was recorded using a Carl Zeiss inverted microscope linked to a Macintosh computer and Macintosh Openlab data acquisition software in a temperature and CO_2 controlled environment. 30 images were captured at 10 second intervals and composite videos produced.

Lymphocytes on the surface of the monolayer were identified by their highly refractive morphology (phase-bright) and rounded or slightly spread appearance. Migrated cells were phase-dark, highly attenuated, and were seen to probe under the EC in a distinctive manner (Greenwood and Calder, 1993; Greenwood *et al*, 1995; Pryce *et al*, 1997).

Control percentage migration was calculated from the percentage of migrated cells as a proportion of the total number of cells per field of view for untreated monolayers. Percentage migration for treatment groups was expressed as a percentage of control. Ten wells were assayed per condition per experiment. Each experiment was performed in triplicate.

2.9 Antibody based assays

Cells were stained to quantitate the percentages of apoptotic cells within differential T cell subsets using antibodies directed to specific T cell markers and cell viability was assessed using a Live, Dead viability assay (see below).

2.9.1 Live, Dead and apoptotic cell staining.

Live cells were characterised by their inherent ability to convert the non-fluorescent precursor Calcein AM to fluorescent Calcein through an intracellular esterase activity. Calcein is a polyanionic dye, which is retained within live cells and produces an intense green fluorescence in live cells, which has a fluorescence excitation wavelength of 495nm and emission at 515nm.

Dead cells were determined by nucleic acid staining with Ethidium homodimer-1, an ethidium bromide analogue, which is able to permeate the cell through the compromised leaky cell membrane. Ethidium homodimer-1 binds to nucleic acids of the dead cells resulting in a red fluorescence, which is excited at a wavelength of 495nm and emits at 635nm.

Apoptotic cells are characterised by the uptake of YoPro-1, a PI derivative (Idziorek *et al* 1995). Early apoptotic cells stain positively for YoPro-1, a monomeric cyanine nucleic acid stain that is capable of penetrating the compromised membrane of an early apoptotic cell. Once inside the cell the dye binds to chromatin bundles characteristic of apoptosis and emits a green fluorescence at approximately 509nm. Early apoptotic cells were defined as cells stained positively for YoPro-1. Late apoptotic cells were defined as cells, which stained positively for both YoPro-1 and EthD-1, however for our purposes were considered to be dead.

The percentage of live, dead and apoptotic cells was quantitated as the percentage of cells positive for a given marker as a percentage of the total T cell population. Subsequent analysis calculated the percentage change in apoptosis using the following equation

$$\frac{(\%\ YoPro^{+}\ \text{cells test population} - \%\ YoPro^{+}\ \text{cells control population})}{\%\ YoPro^{+}\ \text{cells control population}} \times 100$$

2.9.2 Differential T cell subset staining

Differential T cell subsets were identified using a panel of monoclonal antibodies directed at rat T cell surface antigens (see section 2.2). Mouse anti-rat CD5-RPE was used as a marker of rat T cells. Anti-rat CD4-RPE and anti-rat CD8-RPE-Cy5 were used to identify $CD4^{+}$ and $CD8^{+}$ T cells respectively. Mouse anti-rat CD25-RPE specific for the CD25 antigen, the IL-2 receptor α-chain, was used to identify activated T cells. An unconjugated IgG1 mouse anti-rat CD45RC (MRC-Ox22) antibody was used to detect the high molecular mass isoform of the CD45RC antigen (Spickett *et al* 1983). When unprimed, naïve $CD4^{+}$ T cells are stimulated by antigen, the expression of CD45RC changes from a high molecular mass isoform to a low molecular mass isoform. Thus the CD45RC high molecular mass isoform may also

be used as a marker of resting memory T cells. OX-22 was used in conjunction with Star-76, a rat preabsorbed goat-anti mouse IgG-RPE as a secondary antibody.

$2x10^5$ lymphocytes per tube in 100µl PBS were stained with a variety of combinations of antibodies and dyes as shown in Table 2.4. Initially T cells were stained by incubation for 30 min in the dark with 2µl per 10^5 cells for anti-CD5, anti-CD4, and anti-CD25 antibodies and 1µl per 10^5 cells for Star 76 and anti-CD8. Cells incubated with OX-22 were incubated for a further 30 min with Star-76, a rat preabsorbed goat-anti mouse IgG-RPE. Cells were then incubated with 50µl of Calcein AM [0.2µM] (see section 2.4.3), 50µl of EthD-1 [0.1µM] (see section 2.4.8) or 50µl of YoPro-1 [0.5µM] (see section 2.4.29) for 10 min in the dark at 4°C. Cells were then washed twice with 1ml PBS and resuspended in 200µl of PBS for acquisition on a Becton Dickinson FACScan flow cytometer and subsequent analysis using CellQuest analysis software (BD). Gates were set to exclude non-viable cells on forward scatter and side scatter plots, counting at least 5000 events within the gate.

2.9.3 Direct immunofluorescent staining of cells for FasL.

Cell preparation

Both T cells and EC were stained for FasL. $2x10^5$ per sample of rat con A T cell line cells were either unstimulated or stimulated with PMA [25ng/ml] and Ionomycin [1µg/ml] for 6 or 24h. EC were cultured in 6-well plates and either unstimulated or differentially stimulated with IFN-γ [100 U/ml] or IFN-γ [50U/ml] in the presence or absence of TNF-α [5-100U/ml] for 24, 48 or 72h.

To investigate the effects of matrix metalloproteinases upon FasL expression, both T cells and EC were cultured for the duration of the stimulation in the presence of increasing concentrations of KB8301 [0-10µM] an inhibitor of matrix metalloproteinase 7 (a kind gift of Professor Hideo Yagita, Tokyo; Kayagaki *et al* 1995).

FL1	FL2	FL3
YoPro-1*	EthD-1*	-
Calcein*	EthD-1*	-
YoPro -1	CD5-PE	-
YoPro -1	CD4-PE	-
YoPro -1	CD25-PE	CD8-CY5
YoPro -1	Ox22-Star76	-

Table 2.4 Combinations of fluorescent-conjugations used for staining of T cell populations for cell viability and apoptosis on the differential T cell subsets.
*Denotes staining performed upon control T cell populations cultured in the presence of IL-2 but in the absence of EC.

Cell surface staining

Cells were incubated with 40µl biotinylated hamster anti-mouse/rat FasL antibody MFL4 at a range of concentrations [1/10 – 1/400 dilution v/v) in PBS for 30 min on ice in the dark. Cells were then washed with 1ml of PBS and centrifuged at 336g for 4 min. The supernatant was poured off and the cells agitated. Cells were then incubated with 5µl of undiluted streptavidin-PE (SA-PE) and incubated for 30 min on ice in the dark. Cells were again washed with 1ml of PBS and centrifuged at 336g for 4 min. The supernatant was poured off and the cells agitated prior to resuspension in 200µl of PBS and transfer to FACS tubes for acquisition on the BD FACScan flow cytometer. Control untreated EC were stained with 5µl of SA-PE alone to determine non-specific staining of the secondary antibody.

Cells were also double stained for FasL, using ICAM-1 expression as a positive control. Cells were incubated with 50µl of 1A29 anti-ICAM-1 antibody [10µg/ml] for 30 min on ice in the dark and washed with 1ml of PBS prior to incubation with 50µl of donkey anti-mouse IgG-FITC at a 1/100 dilution (v/v) for 30 min on ice in the dark. Control cells were incubated with donkey anti-mouse IgG-FITC alone to determine non-specific binding of the secondary antibody. Cells were then stained for FasL, as described previously, prior to acquisition for flow cytometry.

2.9.4 Intracellular cytokine staining.

In order to permeabilise the cells, cells were incubated with 100µl cytofix/cytoperm for 20 min on ice in the dark. Cells were then washed with 1ml of Permwash by centrifugation at 336g for 2 min. Permwash contains a detergent called saponin, which has the effect of maintaining the pores in cell membranes created by incubation with cytofix/ cytoperm solution. Subsequent steps were carried out in the presence of saponin. Cells were incubated with 40µl biotinylated anti-FasL MFL4 antibody at a range of concentrations [1/10-1/400 dilution v/v] in PBS for 30 min on ice in the dark. The cells were then washed with 1 ml of Permwash before incubation with 5µl of undiluted SA-PE for 30 min on ice in the dark. Cells were washed with 1ml of Permwash and centrifuged at 336g for 4 min. The supernatant was poured off

and the cells resuspended in 200µl of PBS. Cells were transferred to FACS tubes for acquisition on a BD FACScan flow cytometer. Control cells were incubated with SA-PE alone to determine non-specific binding of the secondary antibody.

Cells stained for intracellular FasL were also stained for cell surface ICAM-1. In this situation cells were stained with 1A29 anti-ICAM-1 antibody and donkey anti-mouse IgG FITC as described for cell surface staining. Cells were then permeabilised and stained as described above.

T cells and EC were subsequently stained for cell surface and intracellular FasL using anti-human FasL FSLO1 [1/25 dilution v/v] with 50µl of rabbit anti-mouse IgM µ chain [1/50 dilution v/v] as a secondary antibody and also anti-human FasL 4H9 [10µg/ml] and anti-human FasL 4A5 [10µg/ml] in conjunction with goat anti hamster IgG FITC [1/50 dilution v/v]. The antibody concentrations used were recommended by the manufacturer.

Gates were set to exclude non-viable cells on forward scatter and side scatter plots, and at least 5000 events were analysed within the gate.

2.9.5 Immunocytochemistry and Confocal Microscopy

The anti-rat FasL antibody, MFL4 had not been previously tested for use in immunocytochemistry and confocal microscopy. Cells were stained following a protocol described by Bossi and Griffiths (1999) although a different cell type and antibody were to be used. Cells were stained with 100µl of the MFL4 Ab [10µg/ml] for 30 min at RT in the dark. Cells were then washed three times with 3ml of PBS. Cells were then stained with 100µl of SA-Texas Red [1/100 dilution v/v] for 30 mins in the dark at RT and 100µl of Hoescht [1/100 dilution v/v]. Cells were then washed three times with 3ml of PBS, prior to mounting of cells with MOWIOL. Cells were then analysed using a Zeiss LSM 510 confocal microscope and LSM 5 Image Browser software.

2.10 Mouse soluble Fas ligand ELISA.

A commercially available mouse Fas ligand ELISA was tested to quantitate sFasL production by rat cells due to the 91% homology of mouse and rat FasL at the amino acid level. Rat sFasL concentrations were determined in cell supernatants of BEC and HEV monolayers which were either untreated or differentially stimulated with cytokines at a range of concentrations and combinations. Rat T lymphocyte supernatants stimulated with PMA [25ng/ml] and ionomycin [1μg/ml] for 6 or 24h were used as a positive control. Essentially the ELISA was performed as per the manufacturer's instructions. Briefly, all reagents were brought to RT before starting the experiment. The assay employed a quantitative sandwich enzyme immunoassay, in which a monoclonal antibody specific for mouse FasL had been pre-coated onto the 96-well microplate. Standard curves were generated by 2-fold serial dilution of the supplied mouse Fas ligand standard solution ranging from 0-2000pg/ml. All samples and standards were assayed in duplicate in order to produce statistically significant data. Samples were incubated with the pre-coated plates for 2h whilst agitating at room temperature followed by washing 5 times with wash buffer (supplied). 100μl FasL conjugate was added to each well for 2h. The plate was washed as before, in preparation for the addition of 100μl of substrate solution for 30 min in the dark with gentle agitation to ensure thorough mixing. 100μl Stop Solution, containg 4N NaOH was then added to each well and the optical densities determined using a SpectraCount microplate reader (Beckman). Triplicate readings of each well were taken at 450nm and 540nm to correct for optical imperfections in the plate and sFasL concentrations were normalized to the 0pg/ml standard and calculated using the regression formula of the linearized standard curve. Mean concentrations were calculated from the duplicate wells. The mean lower limits of detection of this ELISA was 3.6pg/ml.

2.11 Reverse transcriptase - polymerase chain reaction (RT-PCR)

2.11.1 RNA extraction

Total RNA was extracted from BEC and HEV monolayers using an RNeasy kit, (Qiagen, UK) according to the manufacturer's instructions. After complete removal of medium from the wells, cells were lysed by addition of lysis buffer RLT containing β-mercaptoethanol (β-ME) [1% v/v]. Cell lysate was passed through a sterile (RNase-free) syringe and 20 gauge needle (Ø.9 mm) 15 times in order to homogenize any high molecular weight DNA. RNA was extracted using the chloroform and ethanol method and air dried before being resuspended in 30μl diethylpyrocarbonate-treated distilled water (DEPC-H_2O).

1μl of extracted RNA was diluted in 70μl DEPC-H_2O in order to determine purity and concentration of RNA by spectrophotometric analysis, using the A_{260}:A_{280} ratio to determine RNA purity, with an absorbance of 1 unit at 260nm corresponding to 40μg RNA per ml. Total RNA was calculated using the following formula:

RNA yield [μg/ml] = A260x40x dilution factor

2.11.2 Complementary Deoxyribose Nuceleic Acid (cDNA) synthesis

2μg of RNA was then reverse transcribed to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions. Briefly, cDNA synthesis was catalysed by superscript II reverse transcriptase using oligo$(dT)_{12\text{-}18}$-primers to hybridise to 3' poly (A) tails. 2 μg total RNA was denatured with 0.5 μg oligo(dT) at 65°C for 5 min and chilled on ice. The reaction mixture, consisting of 10 mM deoxynucleotide triphosphates (dNTP); mix; 10x PCR buffer; 25mM $MgCl_2$; and 10mM DTT; was then added to the RNA/oligo$(dT)_{12\text{-}18}$ primers to make a volume of 19μl. This mixture was then warmed to 42°C for 5 min before addition of 1μl SuperScript II reverse transcriptase and incubated at 42°C for 50 min. Finally, heating at 70°C for 15 min terminated the reaction and the cDNA was stored at –20°C until use.

2.11.3 PCR amplification

2μl aliquots of each cDNA preparation were amplified by PCR. The rat specific primers used for β-actin and FasL were: β-actin (sense: 5'-ATGAGGTAGTCTGTCAGGT-3'; antisense: 5'-ATGGATGACGATATCGCTG-3') (Nudel *et al* 1983) and FasL (sense: 5'-CCCTCTAAAGAAGAAGG-3'; antisense: 5'-AAATGGTCAGCAACGGTAAG-3'), giving 591 and 589 base pair (bp) products respectively. A PCR reaction mixture containing 10 x RT-buffer; 25mM $MgCl_2$; 10mM dNTP mix and 1U Taq DNA polymerase was added to the cDNA/primers to reach a final volume of 50μl and this was overlaid with one drop of mineral oil. PCR conditions optimized for rat β-actin primers were: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles in a DNA thermal cycler. PCR conditions optimized for rat FasL primers were: denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min for 40 cycles. CCl_3 was added to each tube and 10μl of the aqueous phase removed (PCR product). 10μl of the PCR products were then mixed with 10μl loading buffer and resolved by 1.5% agarose gel electrophoresis visualized by ethidium bromide. A 100bp DNA ladder was used as a molecular weight marker and the size of all DNA products corresponded to that predicted. All experiments were performed using cDNA samples positive for rat β-actin. To confirm specificity of the PCR reaction for rat FasL PCR, products from each amplification protocol were isolated and cycle-sequenced on an ABI PRISM automated DNA sequencer and the identity of the amplicon was confirmed using NCBI Genebank.

For quantification of relative band intensities, both FasL and β-actin were confirmed to be in the linear part of the PCR scale by cDNA concentration titration for β-actin and cycle titration for FasL (not shown). Data was obtained using β-actin at a 1/400 dilution of cDNA and FasL at 36 cycles. All positive bands were of the expected size. Relative band intensity was then quantitated using ScanAnalysis v2.50 software and the relative amounts of FasL mRNA expression were calculated as a proportion of the β-actin expression in each sample.

2.12 Western blotting

2.12.1 Cellular lysates for Western blotting

To test whether the FSLO1, anti-human FasL antibody was able to recognise rat FasL, cellular lysates were prepared from unstimulated and stimulated rat T cell cultures as a positive control. $1x10^6$ T cells were set up at $2x10^5$ cells/ml in T cell medium (see section 2.4.26) and either untreated or stimulated for 6 or 24h with PMA [25ng/ml] and ionomycin [1ng/ml]. Cells were harvested by centrifugation and lysed at 4°C for 15 min in 500µl of ice cold lysis buffer containing HEPES [10mM pH 7.5], NP-40 [0.25% v/v], magnesium chloride [1.5mM], sodium chloride [50mM]), EGTA [0.5mM], β-glycerophosphate [50mM], sodium orthovanadate [1mM], PMSF [0.5mM], leupeptin [10µg/ml], aprotinin [10µg/ml] and sodium fluoride [1mM]. The lysate was collected and centrifuged for 10 min at 4°C at 12000g. Protein concentrations were determined using a standard Bradford protein assay and then stored at -70°C.

2.12.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

20µg of protein in SDS sample buffer was added per lane of a 10% polyacrylamide gel. Cellular proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were then transferred by semi-dry blotting to a nitrocellulose membrane.

2.12.3 Immunoblotting

Immunoblotting of FasL protein was attempted using the mouse-anti human FasL Ab clone FSL01 from Biocarta as it had previously been demonstrated to recognise FasL using this method. Although the FSLO1 antibody is a mouse IgM antibody, upon speaking to the manufacturer we tested the cross reactivity of Protein A/G with this antibody using the method below.

Membranes were blocked with 5% Marvel-PBS (M-PBS) for 1h at RT. Membranes were then washed twice with Tween 20/PBS (PBST; 1% v/v). Immunoblotting for

FasL was performed using the mouse anti-human FasL antibody FSL01 [1/25 dilution v/v) in M-PBS for 2h with continuous agitation at RT. Membranes were then washed twice with PBST and incubated with Protein A/G-HRP [1/10000 dilution v/v] in M-PBS. Membranes were washed for 1h and proteins were then visualised by enhanced chemiluminescence (ECL) and X-ray film.

2.13 *In vivo* assays

2.13.1 Induction of Rat EAU

Experimental Autoimmune Uveoretinitis (EAU) was induced using SAg peptide$^{273-289}$ (PNSSLTKTLTLVPLLANNRE; Fling *et al* 1989). EAU was induced in 4-6 week old female Lewis rats by hind footpad (50µl) and base-of-tail immunisation (remainder) with 50-600µg soluble retinal Ag peptide$^{273-289}$ [2mg/ml] emulsified complete Freunds Adjuvant (CFA) supplemented with *Mycobacterium Tuberculosis* (strain H37Ra, final concentration 2.5mg/ml). Animals were also inoculated with $5x10^9$ *Bordetella pertussis* organisms in a 100µl volume intraperitoneally.

2.13.2 Treatment of EAU

The effect of lovastatin on EAU was assessed by *in vivo* administration of lovastatin. Lovastatin was dissolved in DMSO: PBS [1:1 dilution v/v] prior to administration. All animals were anaesthetised with isofluorane prior to intraperitoneal delivery of therapeutics. EAU animals were either treated daily with DMSO:PBS to serve as a vehicle control or treated with lovastatin [20mg/kg/day]. A further group of animals induced with disease were also treated with lovastatin [20mg/kg/day] and twice daily doses of mevalonolactone [2mg/kg] according to previous work by Greenwood *et al* 2003. Mevalonolactone was administered to determine whether addition of exogenous mevalonolactone, a metabolic product downstream of lovastatin action was able to modify the effects of lovastatin. Lovastatin therapy commenced day 6 post-immunisation and continued daily until expected peak of disease (day 15). Non-diseased non-immunised animals served as normal controls.

2.13.3 Fluorescein Angiography (FA)

Clinical progress of control animals and those induced with EAU were visualised by FA. In order to do this, animals were anaesthetized with 0.2ml/100g of a Domitor:Ketamine solution [25%:37.5% v/v]. Pupils were dilated using Tropicamide [1%] and Phenylephrine [2.5%]. Animals were restrained using a mouthpiece and 200µl of Fluorescein [10%] was injected intraperitoneally and the left eye visualized using a scanning laser ophthalmoscope. Saline solution was administered to the cornea during visualization. Early angiograms were captured between 2 and 7 min and late angiograms were captured 8-15 min post fluorescein injection.

Post SLO/FA analysis animals were resuscitated with 0.1ml/100g of Antisedan (20% v/v). Animals were monitored until recovery from anaesthesia. FA was performed in a blinded manner. Grading of vascular disease was performed following the criteria documented in Table 2.5.

2.13.4 Clinical grading of EAU

Experimental animals were graded for the severity of disease daily from day 5 to expected peak of disease and subsequent termination of animals at day 15 or day 22. Clinical examination of eyes was performed by two observers. Table 2.6 details the criteria employed to determine severity of disease induced.

2.13.5 Retinal histology

Animals for which eyes were required for histological analysis were terminally anaesthetized with 500µl of Euthatal. The thorax was opened at the base of the sternum and the diaphragm cut to expose the chest cavity and the lungs. Two incisions were made up the left and right hand side of the chest and then restrained using a clamp. The liver was lifted to reveal the descending aorta, which was subsequently clamped. The thin pericardium was removed from the heart and an incision made into the right atrium. A larger incision was made into the left ventricle just above the point of the heart. A small gavage needle attached to the perfusion

Grade	Fluorescein angiographic signs
0	Normal. No leakage.
1	Mild leakage: hazy but reasonable view of optic disc, optic cup and retinal vessels entering the optic disc.
2	Moderate leakage: unable to identify optic disc and retinal vessels entering the optic disc clearly.
3	Severe leakage: no view of optic disc, optic cup or retinal vessels entering the optic disc.

Table 2.5 Criteria for the grading of vascular disease.
Scoring of severity of vascular disease was performed following the above criteria. Fluorescein angiograms were scored in a blinded manner.

Clinical grade	Clinical signs
0	Normal. Clear medium with good red reflex.
1	Minimal but clearly positive signs of inflammation with occasional cells in Anterior chamber. Dilation of iris vessels.
2	Presence of anterior chamber cells with minimal hypopyon.
3	Diffuse exudates within AC and vitreous plus moderate hypopyon. Dull red reflex. Synechiae.
4	Presence of large cellular and fibrinous exudates. (AC very cloudy). Large hypopyon in AC. Loss of red reflex.
5	Presence of grade 4 with gross orbital oedema and proptosis of globe.

Table 2.6 Criteria for the clinical scoring of EAU.

Clinical scoring of EAU was performed following the above criteria and animals were scored in a blinded manner. Criteria adapted from Merryman *et al* (1991).

pump was inserted into the hole in the ventricle and the needle was inserted high up towards the ascending aorta and clamped in place. The rat was then perfused with 50mls PBS at 15ml/min. The vessels within the chest cavity were observed to ensure perfusion of blood. The animals were then fixed to maximise preservation of the ocular vessels with 35ml Karnovsky's half strength solution pH 7.4 (1% paraformaldehyde and 3% glutaraldehyde in 0.1M sodium cacodylate-HCl). Stiffening of limbs was ensured prior to ceasing fixation. The eyes were enucleated and stored in Karnovsky's fixative until subsequent processing for histological analysis.

Animals whereby tissues in addition to the eyes were required for further cellular analyses were not perfuse-fixed. Following schedule 1 killing, eyes were enucleated, pierced with a 21gauge needle and immersion-fixed in Karnovsky's fixative for at least 24h prior to processing.

2.13.6 Histological preparation of retinal sections.

Segments of eyes perfuse-fixed with glutaraldehyde [3% w/v] and paraformaldehyde [1%] in sodium cacodylate-HCl buffer [0.08M, pH7.4] were rinsed (x3) in PBS and immersed in aqueous osmium tetroxide solution [1% w/v] for 2h at RT. Tissue was then rinsed in distilled water (x3), dehydrated by single 15 min incubations in 50%, 70% and 90% 3 x 100% ethanol, 2 x 20 min changes of propylene oxide and left overnight in a 1:1 mixture of propylene oxide:araldite to infiltrate. This was changed for full resin and left on a rotator for 6h before embedding and overnight polymerization at 60°C. Semithin sections for examination by light microscopy were cut using a Leica ultracut S microtome and diamond knife, stained with a mixture of borax [1%] and toluene blue [1%] in ethanol [50%] at 60°C, dried and mounted in DPX.

All stains and resins were supplied by Agar Scientific Ltd, Essex. All histological preparation was carried out by Dr Peter Munro, Institute of Ophthalmology, UCL.

2.13.7 Histological Analysis

Histological analysis was performed on retinal sections from normal, vehicle, lovastatin and lovastatin and mevalonolactone treated animals. Sections were graded using criteria (Table 2.7) adapted from those previously described by Merryman *et al* (1991). Light micrographs of toluene blue stained histological rat retinal semi-thin sections were taken using a Zeiss LSM 510 confocal microscope operating in transmission mode using a FITC configuration.

2.13.8 Splenocyte Proliferation Assays

Splenocytes were harvested from normal, vehicle, lovastatin treated and lovastatin and mevalonolactone treated rats. Cells were processed (as described in section 2.5.3) and resuspended at $2x10^6$/ml in T cell medium (see section 2.4.26). $4x10^5$ cells were added per well of a flat bottomed 96-well plate and cells incubated in triplicate either unstimulated or stimulated with SAg peptide [5μg/ml or 10μg/ml] or Con A [5μg/ml] in the absence or presence of exogenous lovastatin [1-10μM]. Proliferation of the splenocytes was quantitated when unstimulated cells appeared as resting non-proliferating T cells, by the addition of 1μCi of methyl ^{3}H-thymidine for a further 18h. Cells were then adsorbed to nitrocellulose filters using a cell harvester. Methyl ^{3}H-thymidine incorporation was then measured by β-scintillation counting.

2.13.9 Adoptive transfer of Rat Experimental Autoimmune Uveoretinitis

In order to determine whether Lewis rat SAg peptide$^{273-289}$ primed PLN T cells were capable of inducing disease in naïve animals i.e. uveitogenic, an adoptive transfer model was employed. PLN were isolated from SAg peptide immunised Lewis rats and processed as described previously (see section 2.5.3). T cell lines were established as described in section 2.6.5 and after 6 passages, $1x10^6$ or $5x10^6$ recently activated (24-48h) SAg peptide specific T cells were immunised via tail vein or intraperitoneally into 4-6 wk old Lewis rats. EAU disease was monitored by clinical scoring, SLO/FA and histological analysis as described previously (see sections 2.13.3, 2.13.4, and 2.13.7).

Grade	Histological signs
0	Normal
1	Minimal signs of inflammation. Occasional inflammatory cells in the vitreous base or scattered cells in the neuroretina or adjacent to the ciliary body. V. minor focal retinal detachment generally around optic nerve and occasional focal retinal folding.
2	Uniform inflammatory cell infiltration of the photoreceptor cell layer (PR), outer nuclear layer (ONL) and inner nuclear layers (INL) of the retina and infiltration of the vitreous cortex. Some perivascular cuffing. Retinal damage < 20%
3	Infiltration of the PR layer, ONL and INL of the retina with some retinal detachment (20-50%). Moderate retinal folding, diffuse cellular infiltration of the vitreous, and moderate uveal involvement.
4	Full thickness involvement of the retina, serious retinal detachment and folding. Subretinal exudate. Necrosis and gliosis, substantial uveal involvement
5	Severe retinal damage with destruction of the photoreceptor layer. Large scale retinal folding and large subretinal exudate. Massive cellular infiltrate and loss of anatomical structure.

Table 2.7 Criteria for histological analysis of EAU.

Histological analysis was performed upon toluene blue stained sections by two independent observers following the above criteria adapted from Merryman *et al* 1991.

2.13.10 Induction of mouse EAU

Male B10.RIII (7INS) mice (5-7 weeks old; >19g body weight; Harlan Olac) were immunised bilaterally subcutaneously with 25μg of human IRBP peptide$^{161-180}$ (SGIPYIISYLHPGNTILHVD), emulsified in incomplete Freund's adjuvant supplemented with 60μg/ml Mycobacterium Tuberculosis, as previously described by Hankey *et al* (2001).

2.13.11 Treatment of EAU

The effect of lovastatin and atorvastatin on EAU was assessed by *in vivo* administration of statins. Lovastatin was dissolved in DMSO:PBS (1:1 v/v) prior to administration while atorvastatin was dissolved in PBS. Administration of lovastatin was via intraperitoneal injection while atorvastatin was administered by oral gavage. EAU animals were either immunised daily with DMSO:PBS to serve as a vehicle control for lovastatin treated animals, treated with lovastatin [20mg/kg/day], or following a loading dose of lovastatin [100mg/kg] animals were immunised twice daily with lovastatin [20mg/kg]. A further group of animals induced for disease were also treated with lovastatin [20mg/kg/day] and twice daily immunisations of mevalonolactone [2mg/kg] or squalene [2mg/kg/day]. Animals were also administered with PBS by oral gavage as a vehicle control for animals treated with atorvastatin [10mg/kg/day]. Statin therapy commenced 5 days post immunisation and continued until expected peak disease (day 12; Hankey *et al* 2001). Non-diseased, non-immunised animals served as normal controls.

2.13.12 Fluorescein angiography

Fluorescein angiography (FA) of the right eye was performed using a method previously described (Hawes *et al* 1999). FA was on carried out on 3 normal control animals and, at day 10 post-immunisation, on 7 vehicle treated EAU animals, 6 EAU-induced animals treated with lovastatin [20mg/kg], 6 EAU-induced animals treated with lovastatin [20mg/kg] and twice daily mevalonolactone [2mg/kg] and 6 EAU-induced animals treated with lovastatin [20mg/kg/day] and squalene [2mg/kg/day]. Mice were injected intraperitoneally with sodium fluorescein [2% v/v] at a dose of

0.1ml per 15grams body weight. Pupils were dilated using tropicamide [1%] 20 min prior to examination. Using a Kowa Genesis small animal fundus camera (Tokyo, Japan) photographs of the retina were taken as previously described. The imaging set-up was used in combination with a Volk 90 dioptre condensing lens (Mentor, OH, USA) mounted between the camera and the eye. Images were captured at 3 min and at 5-8 min after the injection of fluorescein on black and white film (Kodak TMAX P3200) in conscious mice.

The fluorescein angiograms were assessed blind by a trained clinician for signs of vascular leakage, hyperfluorescence, and abnormal vascular calibre. Vascular leakage at 3 min after the injection of fluorescein was graded as described in Table 2.5.

2.13.13 Mouse Splenocyte proliferation

Splenocytes were harvested from each of the experimental groups at peak disease (day 12) and prepared as described previously (see section 2.5.3). Cells were resuspended at $5x10^6$ cells/ml in T cell medium (see section 2.4.26) and $1x10^6$ cells were added per well of a flat bottomed 96-well plate in triplicate wells. Cells were either unstimulated or stimulated with $IRBP^{161-180}$ peptide [5μg/ml or 20μg/ml] or Con A [5μg/ml] as a positive control in the absence or in the presence of an anti-MHC class II blocking antibody [0.5μg/10^6 cells]. Proliferation of the splenocytes was assessed at 72h by the addition of 1μCi of methyl ^{3}H-thymidine for the final 18h of culture. Cells were then harvested onto nitrocellulose filter using a Dynatech Cell Harvester, and methyl ^{3}H-thymidine incorporation measured by β-scintillation counting.

2.13.14 Mouse Splenocyte cytokine production

Splenocytes were harvested from each of the experimental groups at peak disease. Cells were processed as described previously (see section 2.5.3) and resuspended at $5x10^6$/ml in T cell medium (see section 2.4.26). $1x10^6$ cells were added per well of a 96-well plate per time point and were either unstimulated or stimulated with $IRBP^{161-180}$ [20μg/ml] or with Con A [5μg/ml]. Cells were cultured for 24-120h when the

cells were harvested and cell supernatants collected by centrifugation of cells at 336g for 4 min and stored at -70°C.

IL-2, 4, 5, IFN-γ, and TNF-α concentrations in the culture supernatant were determined by Cytometric Bead Array analysis and ELISA while IL-10, IL-12, and TGF-β1 were assayed by ELISA alone.

2.13.15 Cytokine ELISAs

ELISAs were carried out following the manufacturers instructions, essentially following the protocol described in section 2.10.

The lower limits of detection for each of the ELISAs were as follows; IL-2, 3pg/ml; IL-4, 2pg/ml; IL-5, 7pg/ml; IL-10, 4pg/ml; IFN-γ, 2pg/ml; TNF-α, 5.1pg/ml and TGF-β, 2.89pg/ml.

2.13.16 Cytometric Bead Array (CBA)

The mouse Th1/Th2 cytometric bead array was used to quantitate IL-2, IL-4, IL-5, IFN-γ and TNF-α secretion by normal, vehicle, lovastatin and atorvastatin-treated splenocytes. The CBA assay was carried out following manufacturer's instructions. Briefly 50μl of capture beads labelled with discrete fluorescence intensities for each of the different cytokines were incubated with splenocyte supernatants (unknowns), recombinant mouse cytokine standards and a negative control. Standard curves were generated over the concentration range of 0-5000pg/ml. 50μl of mouse Th1/Th2-PE detection reagent was added to each of the assay tubes and then incubated for 2h in the dark at RT. Samples were then washed with 1ml of wash buffer (supplied) by centrifugation at 200g for 5 min. The supernatant was then aspirated and 300μl of fresh wash buffer added to each sample tube. Samples were then acquired immediately using a BD FACScan flow cytometer. Acquisition was performed following the manufacturers instructions and analysis was performed using BD CellQuest and BD CBA software.

The lower limits of detection for each of the cytokines using this assay were as follows: IL-2, 5pg/ml; IL-4, 5pg/ml; IL-5, 5pg/ml; IFN-γ, 2.5pg/ml and TNF-α, 6.3pg/ml.

2.13.17 Determination of serum cholesterol

Serum samples were prepared from peripheral blood isolated from EAU mice at peak disease (day 12) by cardiac puncture. Serum samples were stored at -20°C until required for assay when total serum cholesterol was determined spectrophotometrically using the Infinity™ cholesterol liquid stable reagent following manufacturer's instructions.

2.13.18 Determination of statin concentration

Plasma was prepared from peripheral blood isolated from EAU and EAE (see section 2.13.22) mice at peak disease (day 12) by cardiac puncture and stored at -70°C until assay. Supernatants were also harvested from statin-treated EC monolayers and stored at -70°C until required for assay. Active atorvastatin acid and lovastatin hydroxy acid levels were determined in plasma and cell supernatants using liquid chromatography tandem mass spectrometry (LC/MS/MS) by HFL Contract Research (UK). Briefly atorvastatin acid or lovastatin hydroxyacid was extracted using a liquid-liquid extraction procedure followed by chromatographic separation using a reversed phase Phenomenex (Luna C18, 50 x 2.0mm, 5μ) analytical column. The analyte was ionised using an electrospray interface operating in negative ion mode and detection was via tandem mass spectrometry (MS/MS) in the multiple reaction-monitoring (MRM) mode. Simvastatin hydroxy acid was used as the internal standard for both analytes. The bioanalytical assays were sensitive over the concentration range of 0.5-500ng/ml for atorvastatin acid and 1-1000ng/ml for lovastatin hydroxyacid.

2.13.19 Determination of Regulatory T cells

PLN cells were isolated from normal, vehicle or statin treated mice as described in section 2.5.3 and stained for cell surface markers of CD4, CD25 and CD62L (Fisson

et al 2003). $1x10^6$ cells were placed in a 1.5ml centrifuge tube and centrifuged at 336g for 5 min. Cells were then agitated and stained in the dark on ice for 30 min with 50µl of anti-mouse CD25-PE [4µg/ml], 50µl of anti-mouse CD62L-FITC [2µg/ml] and 50µl of anti-mouse CD4-PerCP [20µg/ml]. After washing twice in PBS cells were acquired for flow cytometric analysis. Regulatory T cells were defined as $CD62L^{hi}$ $CD25^+CD4^+$ cells and the percentage of regulatory T cells was determined as a percentage of the total $CD4^+$ T cell population.

2.13.20 Detection of functional regulatory T cells

PLN cells prepared as previously described (see section 2.5.3) from a vehicle treated animal were labelled with CFSE following the method of Angulo and Fulcher (1998). Briefly cells were labelled with CFSE [10µM] by incubation for 10 min in a 37°C waterbath. The labelling reaction was terminated by adding 1ml of cold RPMI containing FCS [10%]. Cells were then centrifuged at 336g for 5 min and resuspended in 1ml of RPMI-1640 supplemented with FCS [10%]. The CFSE labelled cells were allowed to equilibrate for 30 min at RT, recounted and then resuspended at $1x10^6$ cells/ml in T cell medium (see section 2.4.26). $1x10^5$ CFSE labelled responder cells were then added per well to a flat bottomed 96 well plate. Cells were incubated in triplicate either alone to determine constitutive proliferation, in the presence of ConA [5µg/ml] as a positive control or with IRBP peptide [20µg/ml] to determine antigen specific proliferation. Cells incubated in the presence of IRBP peptide were also incubated with increasing concentrations ($1x10^3$ to $2x10^5$) of PLN cells isolated from vehicle, lovastatin treated or atorvastatin treated mice. Antigen-specific proliferation was assessed 5 days post stimulation. Cells were transferred to FACS tubes and immediately prior to acquisition on the flow cytometer labelled with 2µl of PI [1mg/ml]. Subsequent analysis was performed upon $CFSE^+$ PI^- negative cells.

Data was also analysed following the method of Angulo and Fulcher (1998) where the percentage of blasts cells was calculated as the number of blast T cells as a proportion of the total T cell population, as defined using forward scatter and side

scatter. The percentage of divided cells was calculated as the total number of T blasts cells, which displayed less CFSE than the initial undivided $CFSE^+$ T cell population or baseline fluorescence. The mitotic activity was then calculated to account for the loss of fluorescence, which is possible when considering that two cells of a given CFSE intensity arose from a single mitosis of a cell possessing a CFSE intensity immediately greater therefore allowing the number of cell divisions that had taken place to be estimated relative to the starting cell number. The division index was calculated as 100 – mitotic activity divided by the mitotic activity and the weighted division index, a measure of the total number of divisions, was calculated as the division index multiplied by the percentage of divided cells.

2.13.21 Intracellular Cytokine Analysis

PLN cells were isolated at peak disease from normal, vehicle or statin treated animals. PLN cells were prepared as described previously (see section 2.5.3) and set up at $2x10^6$/ml. $4x10^5$ cells plated out per well of a flat bottomed 96 well plate. Cells were either unstimulated or stimulated with $IRBP^{161-180}$ [20μg/ml] or MOG^{35-55} [20μg/ml] peptide for 96h, with Brefeldin A (BFA; [10μg/ml]) added for the final 18h of culture. Cells were permeabilised and stained as described previously (see section 2.9.4) with rat anti-mouse IL-4-PE [0.5μg/10^6 cells] and rat anti-mouse IFN-γ-FITC [0.5μg/10^6 cells]. Cells were subsequently analysed by flow cytometry.

The IL-4:IFN-γ ratio was calculated as the percentage of $IL\text{-}4^+$ cells as a percentage of the IFN-γ^+ cells

2.13.22 Induction of mouse EAE

EAE was induced in 8-12 week old female C57BL/6 mice by subcutaneous immunisation with 100μg myelin oligodendrocyte glycoprotein peptide $^{35-55}$ (MOG^{35-55}) emulsified in complete Freund's adjuvant supplemented with Mycobacterium tuberculosis H37Ra [4mg/ml]. Animals were also injected with 100ng Bordetella pertussis toxin on the day of immunisation and 48h later (Youssef *et al* 2002).

2.13.23 Treatment of EAE

The effect of atorvastatin therapy upon EAE was assessed by *in vivo* administration of atorvastatin. Atorvastatin was dissolved in PBS prior to administration. EAE animals were treated daily with PBS as vehicle control or with 10mg/kg/day atorvastatin administered by oral gavage. Treatment commenced within one day of clinical signs of disease being observed (Youssef *et al* 2002) until expected peak of disease at day 17.

2.13.24 Clinical grading of EAE

Mice induced with EAE were examined daily for clinical signs of EAE. Grading was performed following the criteria (Table 2.8) described by Youssef *et al* (2002).

2.13.25 Splenocyte proliferation for EAE

Splenocytes were isolated and prepared as described for EAU animals. Proliferation of splenocytes in response to MOG^{35-55} peptide [20μg/ml] was assessed as previously described (section 2.13.13).

2.13.26 Splenocyte cytokine production of EAE

Splenocytes were isolated and prepared as described for EAU animals. Cytokine production of splenocytes was assessed in response to MOG^{35-55} [20μg/ml] as previously described (section 2.13.14)

2.13.27 Statin Plasma concentrations

Statin plasma concentration was determined in plasma samples obtained from EAE induced animals as described in section 2.13.18.

2.14 Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test for non-parametric analysis and the Students T test for analysis of parametric data. A p value of <0.05 was considered significant while a p value of <0.01 was considered highly significant.

Clinical Grade	Clinical symptoms
0	No disease
1	Limp tail
2	Impaired righting reflex
3	Partial hindlimb paralysis
4	Complete hindlimb paralysis
5	Moribund or dead

Table 2.8 Criteria for the grading of clinical EAE.
Clinical EAE was graded following the criteria described above (Youssef *et al* 2002).

Chapter 3

Induction of T cell apoptosis by CNS EC

Introduction

Apoptosis or programmed cell death is essential for normal development, maintenance of homeostasis and for the elimination of autoreactive T cells. A genetically controlled cell suicide programme (Ellis and Horvitz, 1986, Vaux *et al* 1988), it can be induced by a number of signals both extracellularly and intracellularly. Evidence suggests that death-inducing signals may all be elicited via a common death effector pathway (Vaux and Strasser, 1996). A family of proteins called caspases mediate apoptosis of a cell. Apoptosis can be induced by stimulation of those members of the TNF receptor family which have a death domain (e.g. CD95), or by a broad range of stimuli including growth factor withdrawal. These pathways to effector caspase activation are distinct. Caspase-8 mediated activation of the pro-apoptotic BH3-only protein Bid may provide a link between these pathways in some cell types.

Morphological changes occur during apoptosis, which are characteristic of this specialised form of cell death. During apoptosis nuclear chromatin condenses and cytoplasmic shrinkage and plasma membrane blebbing occurs. Apoptotic cells can be detected by a number of means including terminal dUTP nick end labelling: TUNEL which detects DNA breaks (Gavrieli *et al* 1991); Annexin V detects cell surface exposed phosphatidyl serine (Koopman *et al* 1994); and fluorescent dyes which bind to chromatin bundles e.g. YoPro (Idziorek *et al* 1995).

T cell effector functions include proliferation in response to antigen. In the absence of control mechanisms, continuous proliferation in response to antigen would result in expansion of the immune system. However this is not generally observed which suggests that mechanisms exist to maintain immune system homeostasis. Apoptosis is key to the regulation of T cell homeostasis by means of three main processes. These are activation induced cell death (AICD), cytokine deprivation induced apoptosis and stromal cell mediated rescue of cytokine deprivation induced apoptosis. Optimal antigenic stimulation of T cells results in proliferation, whereas overstimulation of T cells results in the death of the T cell by the process of AICD.

AICD is thought to occur to protect against excessive immune stimulation and is therefore triggered as a result of the re-ligation of the T cell receptor (TCR) upon activated T cells. This form of cell death is generally encountered in the presence of large numbers of T cells and large quantities of antigen (Abbas, 1996) and is known to be Fas-FasL dependent. Interferon-α has been shown to increase AICD through the upregulation of Fas and Fas Ligand (FasL; Kaser *et al* 1999).

In addition to being sensitive to levels of antigen, activated T cells are highly sensitive to cytokines. Activated T cells require IL-2 for continuous proliferation. Omission of IL-2 from T cell cultures *in vitro* results in the phenomenon of IL-2 deprivation induced apoptosis, which can be rescued by reintroduction of IL-2 (Akbar *et al* 1996). IL-2 has been shown to be essential for the prevention of apoptosis in T cell cultures by the up-regulation of apoptosis inhibitory proteins such as Bcl-2 (Akbar *et al* 1993). In addition to IL-2, cytokines such as IL-4, IL-7 and IL-15, which signal through the γ-chain of the IL-2 receptor (IL-2R) are thought to rescue T cells from cytokine deprivation induced apoptosis *in vitro* (Akbar *et al* 1996).

In the absence of IL-2R γ chain interaction, activated T cells are able to survive as a result of interaction with fibroblasts, epithelial and endothelial cells. These cells are able to prevent apoptosis in a process called stromal-cell-mediated rescue (Akbar *et al* 1993). Although this was shown to rescue T cells from apoptosis it was thought that T cells persisted in a quiescent state. It has since been proposed that inhibition of apoptosis by the stromal cell microenvironment is in part responsible for the enhanced survival of inflammatory cells within the joint resulting in chronic inflammation such as rheumatoid arthritis (Salmon *et al* 1997). Subsequent research has shown that this is mediated by synovial fibroblast and macrophage production of type I interferons such as IFN-α and IFN-β and the upregulation of Bcl-x_L in the rescued cell (Pilling *et al* 1999). In addition to the role of the synovium in the persistence of inflammatory cells, chemokine production within the local environment is thought to contribute to the inappropriate retention of T cells within the rheumatoid joint (Buckely *et al* 2000). Stromal derived TGF-β has been shown to

result in the sustained expression of CXCR4 once within the synovial environment which, upon interaction with SDF-1, in turn facilitates a strong integrin-mediated adhesion and thus retention within the rheumatoid joint.

In addition to T cell regulation by cell death T cells can also be rendered in a state of anergy or unresponsiveness (reviewed by Jenkins, 1992), which is achieved by the partial or inappropriate activation of T cells upon presentation with antigen by non-professional APC. This occurs due to a failure to present antigen with costimulation by co-stimulatory molecules such as B7-1 and B7-2 (Slavik *et al* 1999) resulting in cells which are alive but are unable to produce IL-2 and fail to respond to optimal antigenic stimulation (James *et al* 2003).

EC lining the microvasculature of the CNS form the highly active interface between the CNS and the immune system. This interface is the BBB or BRB. Initially thought to prevent immune cell entry to the CNS, it has been shown that activated lymphocytes are able to migrate across the BBB and BRB (Hickey *et al* 1991, Greenwood and Calder 1993, Greenwood *et al* 1995). CNS EC have since been shown to play an active role in the regulation of immune cell entry to the CNS through the regulated expression of cell adhesion molecules (Male *et al* 1990, Pryce *et al* 1991, Greenwood *et al* 1995).

Under normal physiological conditions the central nervous system (CNS) and the retina are "immune privileged" sites. Numerous mechanisms are implicated in the maintenance of immune privilege including the generation of an immunosuppressive microenvironment by the local production of cytokines and neuropeptides (Streilein 1995). However it has been shown that T cells which enter the CNS do not leave but die via apoptosis, and that this process may contribute to the subsidence of disease (Pender *et al* 1992). Previously it has been shown that brain microvessel EC are capable of regulating T cell function by the induction of anergy (Bourdoulous *et al* 1995) and RPE of the posterior BRB have the ability to induce T cell apoptosis (Jorgenssen *et al* 1998). Astrocytes and microglia of the CNS have also been

implicated in the induction of apoptosis of infiltrating inflammatory cells (Weber *et al* 1994, Ford *et al* 1996). Given the increasing understanding of the role of BEC in the regulation of T cell infiltration into the CNS we set about to investigate the role of BEC in the immunoregulation of T cell function.

Aims

To investigate the effects of transmigration across BEC upon the regulation of T lymphocytes.

Results

T cell viability is maintained for up to 72h.

Initially, experiments were carried out to determine basal viability of the T cells used in each of the assays for the duration of the assay. Control T cell populations were cultured in the presence of IL-2 for 24-72h and stained with combinations of calcein AM, EthD1 and YoPro-1 for the determination of cell viability, death or apoptosis, as described in chapter 2 (section 2.9.1 and 2.9.2). Live cells were determined as calcein$^+$ EthD1$^-$, dead cells were determined as cells which stained positively for EthD1, while apoptotic cells were determined to be YoPro-1$^+$ EthD1$^-$ cells. YoPro-1$^+$ EthD1$^+$ cells were determined to be late apoptotic and for the purposes of this study were classified as dead cells. Our results clearly show that T cell viability of a highly activated T cell line could be maintained for the duration of the assay with greater than 94% viability at all timepoints investigated (Figure 3.1). Less than 10% of dead and apoptotic cells were quantitated at each of the timepoints investigated (Figure 3.1).

Percentage recovery of viable CD5$^+$ T cells is greater from HEV EC cultures than BEC cultures.

Given our previous finding we investigated the effects of TEM upon T cell viability. The BD Trucount assay was used to quantitate the recovery of viable CD5$^+$ T cells, which migrated across BEC and HEV monolayers (see chapter 2, section 2.8.4). $3x10^5$ T cells were added to both BEC and HEV monolayers cultured upon transwell inserts with pores of 3.0μM. Significantly greater numbers of viable CD5$^+$ T cells were recovered from HEV cultures as compared with BEC cultures at both 24 and 48h ($p \leq 0.01$; Figure 3.2). This data suggests that T cell viability post TEM is supported by HEV cells but to a lesser degree by BEC monolayers.

CD4$^+$ T cell viability is significantly decreased as a result of migration across BEC monolayers.

Our data suggested that viability of CD5$^+$ T cells is modulated as a result of TEM through BEC monolayers as compared to HEV monolayers. We therefore decided to

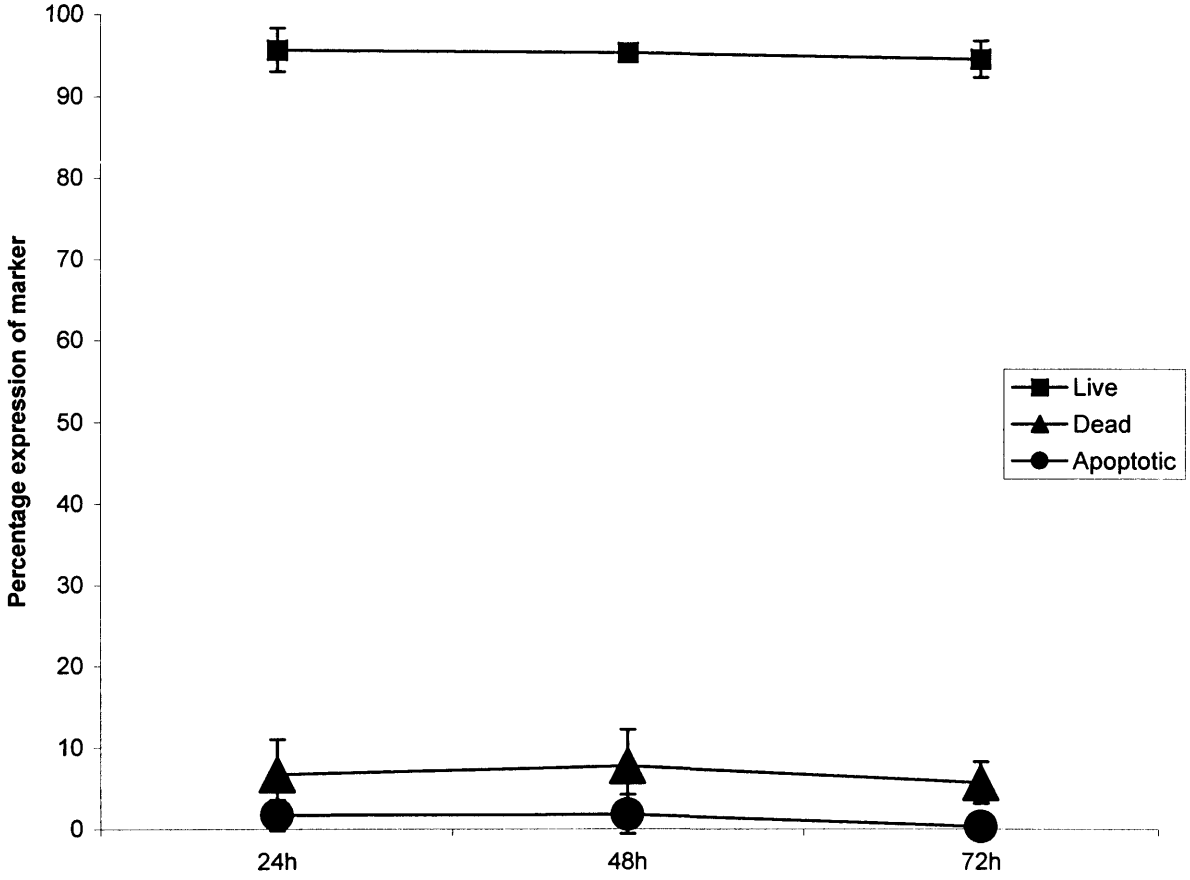

Figure 3.1 Percentages of live, dead and apoptotic cells as a percentage of the total T cell population in control T cell cultures.
T cells were cultured in the presence of IL-2 for up to 72h. Cell viability was then determined by staining of cells with calcein AM as a marker of live cells; ethidium homodimer-1 (EthD1) as a marker of dead cells and YoPro-1 as a marker of apoptotic cells. Results are shown as the means of three independent experiments ± S.D.

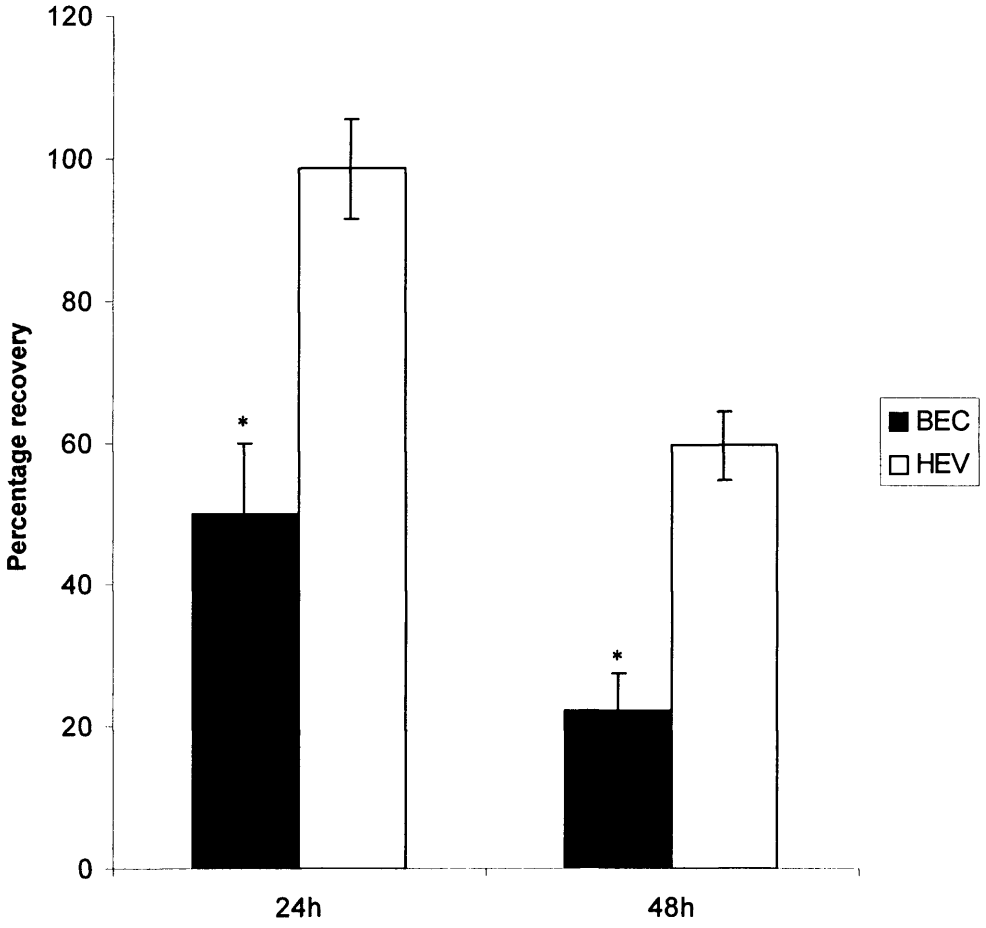

Figure 3.2 Percentage recovery of viable migrated T cells as a percentage of the total number of T cells at the onset of the assay.
T cells were added to EC monolayers and the percentage of viable migrated T cells recovered from the lower assay chamber was quantitated using the BD TruCount assay. Data is shown as the means of four samples ± SEM, except for BEC at 48h which is the means of three samples ± S.D. Statistical analysis was performed using the students T test. *$p \leq 0.01$.

further investigate the effects of TEM upon $CD4^+$ and $CD8^+$ T cells (see section 2.8.3 and 2.9.2).

$3x10^5$ syngeneic T cells were added to BEC and HEV monolayers and at increasing timepoints T cell viability in the $CD4^+$ and $CD8^+$ migrated T cell population was determined. The total percentage of the different subsets was similar in each of the T cell populations: 85.1 ± 2.1% were $CD4^+$ and 7.8 ± 0.6% were $CD8^+$ (mean ± SEM of 7 exp; data not shown). Dual staining of T cells for YoPro-1 as a marker of apoptosis and anti-CD4 or anti-CD8 antibodies revealed an intriguing difference in the levels of YoPro-1^- i.e. live cells (Figure 3.3A). Control $CD4^+$ and $CD8^+$ T cells showed no significant difference in the levels of YoPro-1^- cells at each of the timepoints investigated. However a significant decrease in the levels of YoPro-1^- $CD4^+$ T cells ($p \leq 0.05$) was observed in T cell populations which had migrated through BEC monolayers by 24h (Figure 3.3B). No significant differences were observed in the levels of YoPro-1^- $CD8^+$ T cells which had migrated through BEC monolayers or in the $CD4^+$ and $CD8^+$ T cell population which had migrated through HEV monolayers by 24h. Similarly no significant difference in the level of YoPro-1^- cells was detectable in either the $CD4^+$ or $CD8^+$ T cell population which had migrated through BEC or HEV monolayers by 48h.

TEM across BEC but not HEV monolayers results in a significant increase in $CD4^+$ and $CD8^+$ T cell apoptosis.

As a result of these findings, we continued to investigate the changes in the levels of apoptosis as compared to controls in these populations as described in chapter 2 (section 2.9.1). Due to the inherent variation between biological assays, the percentage change in apoptosis was calculated relative to the control in each experiment. Apoptosis of the control $CD4^+$ T cell population was found to be 14.0 ± 3.7% and 12.5 ± 2.2% at 24 and 48h respectively while levels of apoptosis within the $CD8^+$ T cell population was found to be 4.0 ± 1.0% and 4.4 ± 1.0%. $CD4^+$ T cells which migrated through BEC monolayers by 24h exhibited a significant increase in the levels of apoptosis ($p \leq 0.01$; Figure 3.4), while $CD8^+$ T cells which migrated

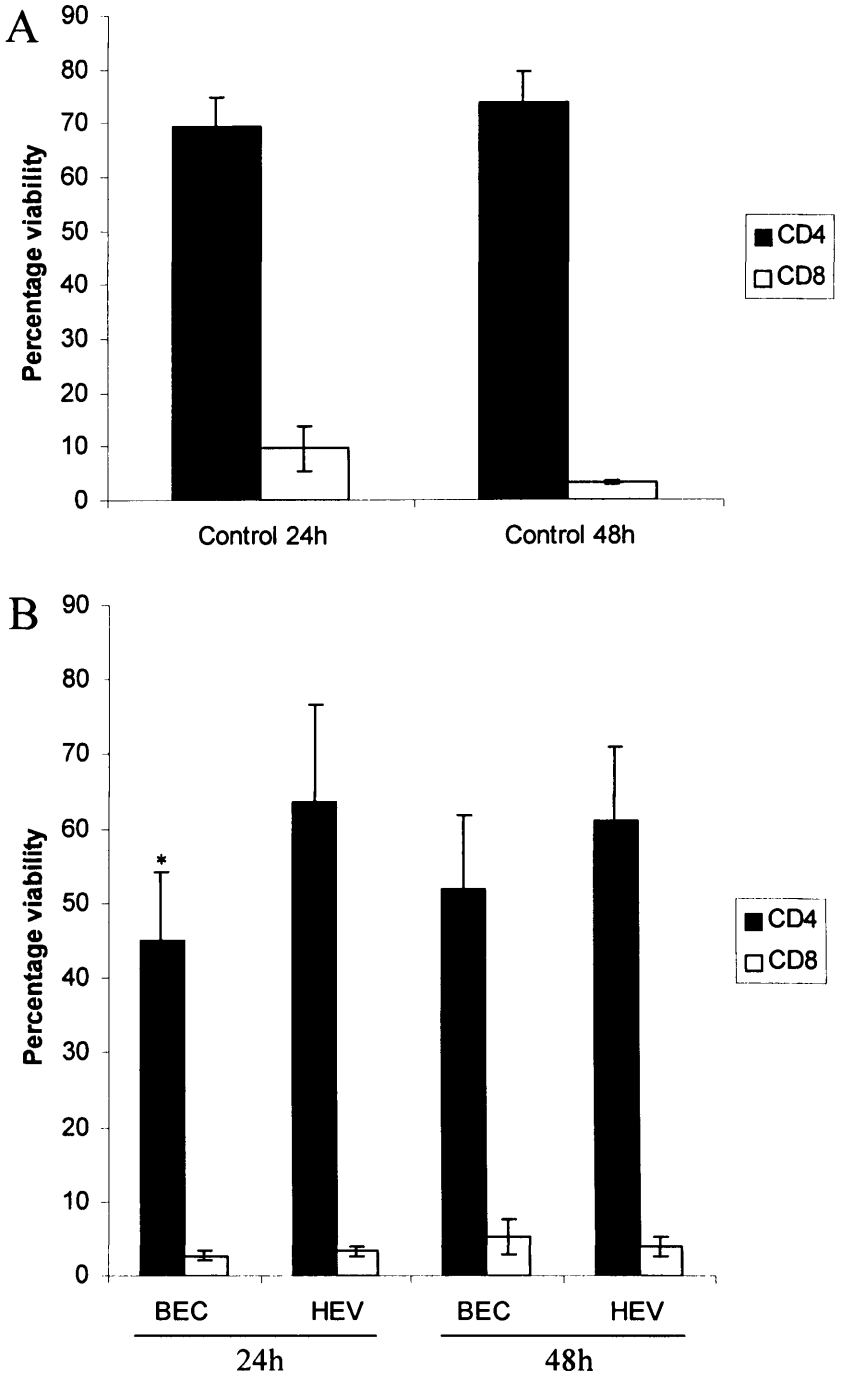

Figure 3.3. showing the percentage of viable T cells in control T cell and migrated T cell populations at 24 and 48h.
Cell viability was determined as the percentage of YoPro$^-$ CD4$^+$ or CD8$^+$ cells as a percentage of the total T cell population in A) control T cells and B) migrated T cells. Values are expressed as the means ± SEM of 7 independent experiments. Statistical analysis was performed using the Students T test *$p \leq 0.05$.

through BEC monolayers by 48h showed a smaller but significant increase in apoptosis ($p \leq 0.05$; Figure 3.4). In contrast to this is the observation that both $CD4^+$ and $CD8^+$ T cells which migrated through HEV monolayers exhibited a decrease in the levels of apoptosis as compared to controls, although these values failed to reach significance (Figure 3.4).

BEC monolayer induced apoptosis is cell contact dependent.

The previous experiments clearly demonstrate that T cells cultured in the presence of BEC exhibit a significant increase in the level of apoptosis as compared to control T cells. However from this data we were unable to deduce the mechanism by which this induction of apoptosis occurs. We therefore set about to determine whether this induction of apoptosis was as a result of direct cell-cell contact or mediated via a soluble factor. In order to investigate this, T cells were cultured in the presence of both BEC and HEV monolayers in the absence of cell-cell contact using a transwell insert system with 0.4μM pores thereby preventing migration of the T cells to the lower chamber. In these cultures T cells and EC share the same culture medium whilst remaining spatially isolated to determine whether direct cell-cell contact is required for the induction of apoptosis. $CD5^+$ T cells cultured in the presence of BEC monolayers for up to 72h showed no significant change in the levels of apoptosis as compared with controls. Interestingly $CD5^+$ T cells cultured in the presence of HEV monolayers exhibited a significant decrease in the levels of apoptosis observed at 24 and 48h ($p \leq 0.01$ and $p \leq 0.001$ respectively; Figure 3.5). A decrease in apoptosis was also observed at 72h but did not achieve significance. This data is consistent with that of the transmigration experiments, in that T cells co-cultured with HEV monolayers exhibit a decrease in the levels of apoptosis of $CD4^+$ and $CD8^+$ T cells (Figure 3.4). This data also supports the ability of HEV EC to protect T cells from apoptosis, by means of a soluble factor. In addition, this data suggests that the increased levels of apoptosis observed in $CD4^+$ and $CD8^+$ T cells which migrate through BEC monolayers as determined by our transmigration experiments is mediated by direct cell-cell contact.

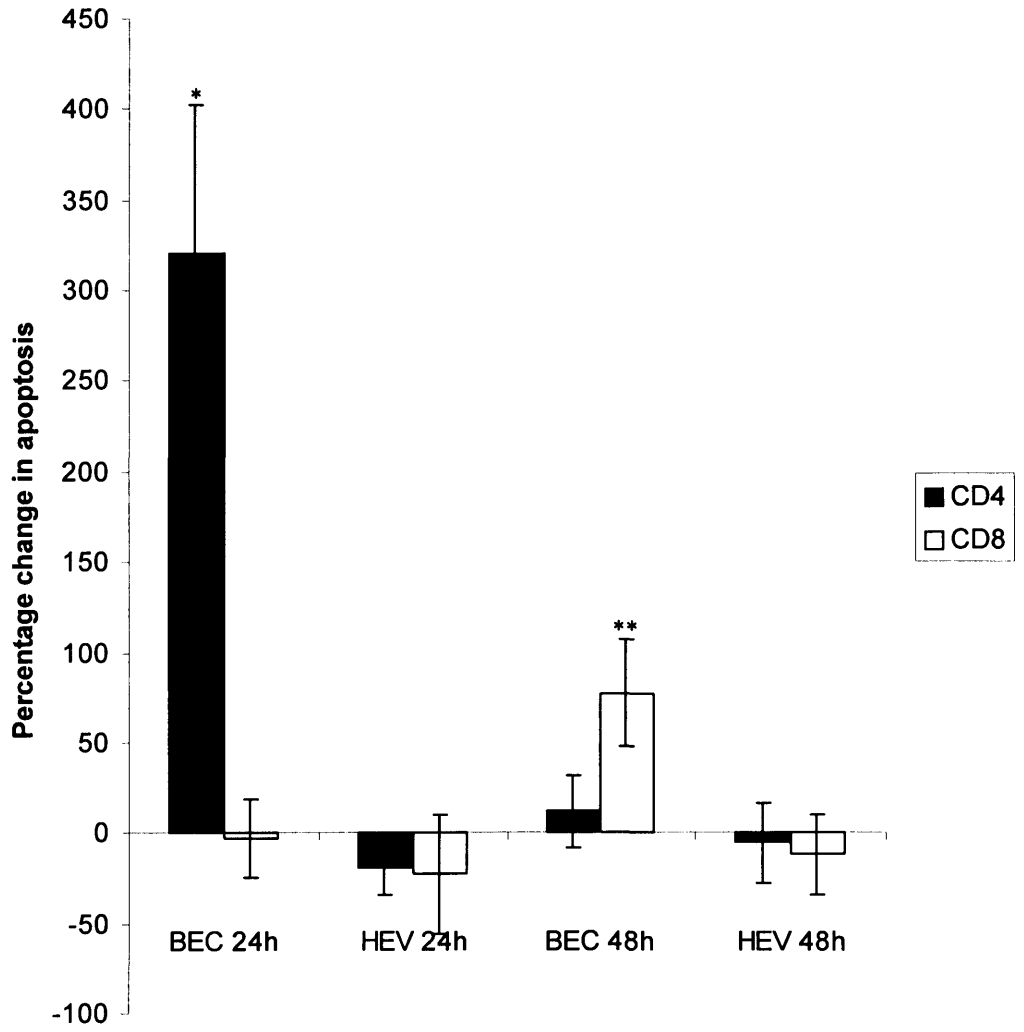

Figure 3.4 Percentage change in the levels of apoptosis of migrated $CD4^+$ and $CD8^+$ T cells.

Levels of apoptosis were determined by YoPro-1 staining. Due to the inherent variation between biological assays, the percentage change in apoptosis was calculated relative to the control in each experiment. A significant increase in apoptosis was observed in $CD4^+$ and $CD8^+$ T cells which migrated through BEC monolayers at 24 and 48h respectively. Values are expressed as the mean ± SEM of at least 4 independent experiments. Statistical analysis was performed using the Students T Test. * $p \leq 0.01$ and ** $p \leq 0.05$).

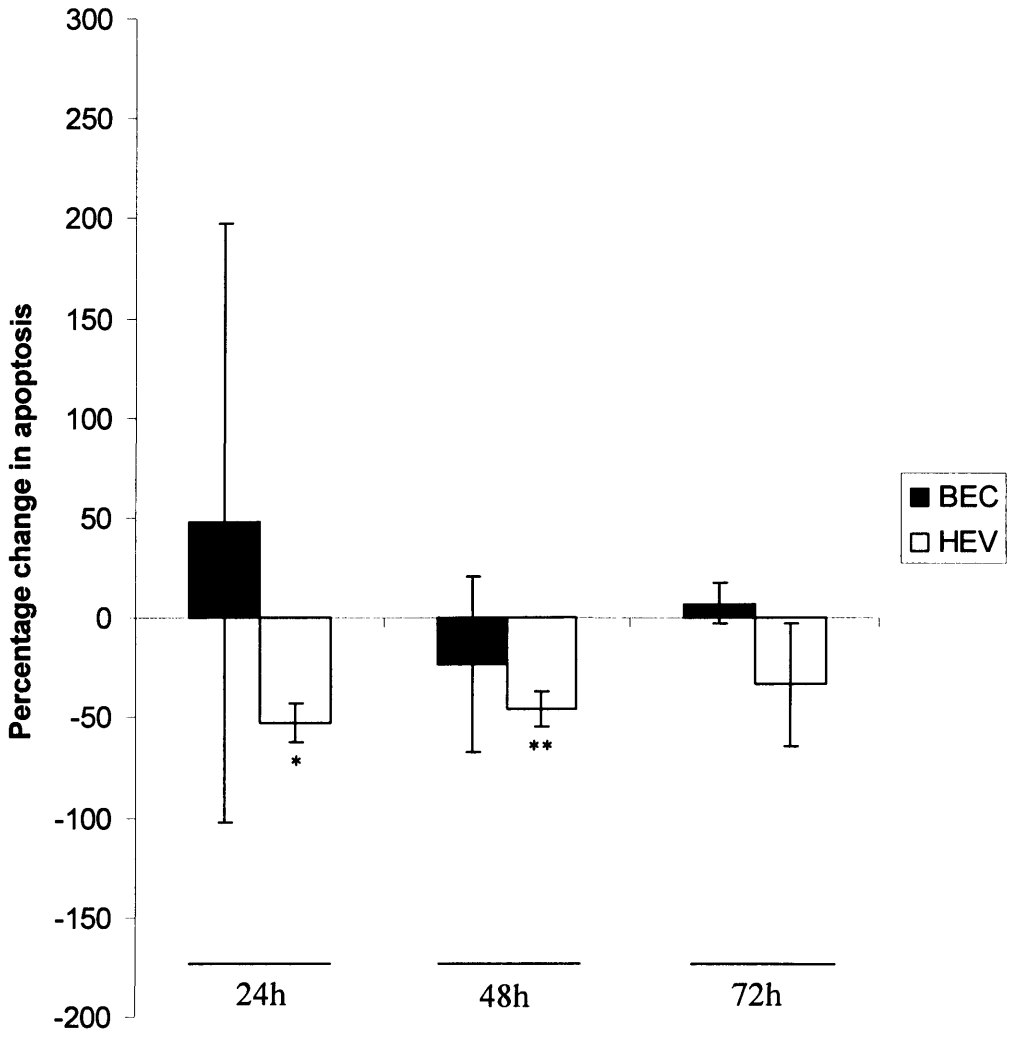

Figure 3.5 showing the change in apoptosis of $CD5^+$ T cells in the absence of cell-cell contact.

Levels of apoptosis were determined by staining with YoPro-1. Due to the inherent variation between biological assays, the percentage change in apoptosis was calculated relative to the control in each experiment. A small but significant reduction in the levels of apoptosis were observed in $CD5^+$ T cell populations cultured in the presence of HEV monolayers for 24 and 48h. Results are shown as the means of three independent experiments ± S.D. Statistical anlaysis was performed using the Students T Test. *$p \leq 0.01$, **$p \leq 0.001$.

Discussion

The CNS is known to be an immune privileged site which is protected from the harmful effects of the immune system by the presence of highly specialised mechanisms such as immune privilege. Initially it was thought that the CNS was devoid of lymphocyte traffic. However, it has since been reported that surveillance of the CNS occurs by activated T cells (Hickey *et al* 1991, Greenwood and Calder 1993).

I therefore investigated the effect of TEM upon T cell viability following migration across BEC monolayers as compared to HEV monolayers. T cells which migrated through HEV monolayers showed significantly greater levels of viability as compared with T cells which migrated through BEC monolayers at each of the timepoints investigated, suggesting that HEV EC maintain T cell viability as a result of TEM. In addition the results observed suggest these effects are as a direct consequence of culture with BEC and HEV monolayers, given that control T cell viability is maintained for up to 72h under similar conditions.

We continued by investigating the effects of TEM upon $CD4^+$ and $CD8^+$ T cells. Our data showed that a significant reduction in $CD4^+$ T cell viability occurred as a result of migration through BEC monolayers. Subsequently we were able to demonstrate that this decrease in viability was associated with a corresponding increase in apoptosis. A significant increase in apoptosis was also detectable in $CD8^+$ T cells, which were found to have migrated across BEC monolayers by 48h. The increase in $CD8^+$ T cell apoptosis did not appear to be associated with a corresponding decrease in $CD8^+$ T cell viability; however this may be a limitation of the assay given the small proportion of $CD8^+$ T cells within the T cell population and the variation between experiments.

As a result of our findings mechanisms by which the induction of apoptosis occurred was investigated. Complementary experiments were set up to determine whether T

cells exposed to BEC and HEV monolayers in the absence of direct cell-cell contact were susceptible to apoptosis. These experiments revealed that direct cell-cell contact is required for BEC induced T cell apoptosis. This data also revealed that T cells cultured in the presence of HEV monolayers were protected from apoptosis in the absence of direct cell-cell contact and thus supports the role for soluble factors in this process.

This data complements that described previously for the functional role of HEV EC and BEC. HEV EC function to maximise lymphocyte migration into the peripheral lymph node (PLN; Girard and Springer 1995) while endothelia of the CNS are adapted to minimise leukocyte trafficking through the low level expression of the requisite molecules for the purpose of migration (Male *et al* 1990, Pryce *et al* 1991, Fabry *et al* 1994).

Our findings presented here are extremely interesting given the role of T cells in immune mediated diseases of the CNS such as multiple sclerosis (MS) and uveitis. This data suggests that BEC may play a role in the regulation of T cell function within the CNS by the induction of apoptosis of both $CD4^+$ and $CD8^+$ T cells as a result of TEM. The temporal separation observed in the induction of apoptosis of $CD4^+$ and $CD8^+$ T cells may reflect the ability of each T cell subset to synthesise and correctly display the requisite molecules upon their surface in order to mediate TEM, although our present studies are unable to confirm this. Alternatively it may be possible that the duration required in order for a $CD8^+$ T cell to be recognised as apoptotic by YoPro-1 is greater than that of $CD4^+$ T cells, hence we observed levels of apoptosis at 48h in $CD8^+$ T cells. This data is suggestive of a greater susceptibility of $CD4^+$ T cells to apoptosis than $CD8^+$ T cells. However since $CD4^+$ T cells are thought to be the initial cells found at MS lesions, this data may reflect *in vivo* findings whereby $CD8^+$ migration may occur post $CD4^+$ migration into the CNS. The significant induction of apoptosis in both $CD4^+$ and $CD8^+$ T cells may, in part, aid in the maintenance of immune privilege within the CNS.

Evidence exists to suggest that the retinal pigment epithelium (RPE) which forms the posterior BRB is capable of inducing apoptosis in T cells (Jorgenssen *et al* 1998) and lymphocytes which enter the mouse eye have been shown to undergo apoptosis (Griffith *et al* 1995). It is therefore plausible to suggest that a similar mechanism may exist at the level of the BBB. Previous studies have demonstrated that brain microvessel endothelial cells are capable of inducing anergy in syngeneic T cells (Bourdoulous *et al* 1995).

In contrast to BEC, HEV EC do not appear to induce apoptosis in either $CD4^+$ or $CD8^+$ T cells when cultured in the absence or presence of direct cell-cell contact at the timepoints investigated. Interestingly it would appear that HEV EC may protect T cells from apoptosis. As mentioned previously this may be due to the functional role of HEV, to maximise lymphocyte migration into the PLN. This data is also consistent with that previously described by Borthwick *et al* (2003) in which transmigration across HUVEC monolayers and HUVEC conditioned supernatants conferred protection against apoptosis. It has also been shown that HUVEC monolayers suppressed T cell proliferation but did not render T cells unresponsive (Marelli-Berg *et al* 1999).

Apoptosis of T lymphocytes is known to occur during EAE (Pender *et al* 1992, Schmied *et al* 1993) which is in contrast to inflammatory disorders of the skin where less apoptosis is observed (Schneider *et al* 1996). It is reported that MS disease activity correlates with enhanced T cell expression of survivin, an anti-apoptotic protein (Sharief *et al* 2002) and that recovery of EAE is associated with a decrease in the survival of encephalitogenic T cells (Chang *et al* 2003). One current therapy for the treatment of MS, IFN-β, is thought to mediate induction of apoptosis of peripheral immune cells (Gniadek *et al* 2003). It has been proposed that astrocytes (Weber *et al* 1994) and microglia (Ford *et al* 1996) of the CNS, acting as non-professional antigen presenting cells (APC's), prime invading T cells for an apoptotic stimulus from the local microenvironment. Given the immune privilege nature of the CNS, this may be due to the presence of local immunosuppressive cytokines or neuropeptides.

Previous findings within our laboratory demonstrate that primary rat BEC and REC fail to support T cell proliferation (Wang *et al* 1995), it is possible therefore that BEC may also function to prime T cells for additional apoptotic signals. However the effects observed within our co-culture would suggest that BEC are capable of inducing apoptosis of T cells in the absence of non-BEC derived environmental stimuli.

Pro-inflammatory cytokines play a critical role in the pathogenesis of MS and EAE. It is known that highly activated T cells secrete such pro-inflammatory cytokines and that BEC respond to such stimuli by the upregulation of cellular adhesion molecules. Therefore it would be extremely interesting to investigate the modulation of T cells as a result of migration across an activated endothelium.

In keeping with the growing understanding of the role of CNS EC in leukocyte TEM, this data supports a role for specialised mechanisms at the BBB, and provides further evidence that endothelia from different vascular beds exhibit specialised properties dependent upon their location (Oh *et al* 2004). This therefore highlights one reason why caution should be taken to apply principles determined for one type of endothelium and bestow such principles upon other endothelia.

The necessity for cell-cell contact suggests close communication between T and EC cells thus suggesting a role for a cell surface mediator of apoptosis. Apoptosis has previously been shown to play a role in the preservation of immune privilege of the eye by the downregulation of T cells (Griffiths *et al* 1995). Apoptosis can be induced by a number of means but due to the dependency of cell-cell contact this suggests mediation of apoptosis occurs via a cell surface initiator of apoptosis and expression of the reciprocal death receptor upon the apoptosis susceptible cell. Activated T cells are known to express Fas which, upon ligation with its ligand (FasL), results in the death of the Fas expressing cell. Considering the aforementioned role for the Fas-FasL interaction in immune privilege (Griffiths *et al* 1995) and in normal T cell homeostasis it seems plausible that this interaction may indeed play a role in T cell

regulation at the level of the BBB. It is also recognised that the BRB and BBB share numerous similarities. We therefore propose that FasL expression by BEC may induce apoptosis of infiltrating inflammatory cells and aid in the maintenance of immune privilege.

Chapter 4

Expression of FasL

Introduction

FS7-associated surface antigen or Fas (APO-1/CD95), first discovered due to its cytocidal properties (Trauth *et al* 1989, Yonehara *et al* 1989), is a 45kD cell surface molecule which is a member of the Tumor necrosis factor-receptor (TNF-R) superfamily (Itoh *et al* 1991). This type I membrane protein is composed of an extracellular domain of three cysteine rich domains, a transmembrane domain and a death inducing cytoplasmic domain which bears significant homology to TNF-R1 (Itoh *et al* 1991, Oehm *et al* 1992). Triggering of the apoptotic pathway requires crosslinking of Fas with either antibodies to Fas, cells expressing FasL or purified FasL (Nagata and Golstein, 1995). It has also been shown that protection against Fas-mediated apoptosis can occur by means of a soluble Fas molecule (Cheng *et al* 1994).

Activation of Fas results in the aggregation of the intracellular death domain, the recruitment of FADD (Fas associating death domain protein/MORT-1) and subsequent crosslinking via its death domain (Boldin *et al* 1995, Chinnaiyan *et al* 1995) which then results in the recruitment of caspase 8 (FLICE/MACH-1/Mch5) (Boldin *et al* 1996, Fernandes-Alnemri *et al* 1996, Muzio *et al* 1996) via its N-terminal death effector domain. Crosslinking of these two key regulatory proteins to Fas forms the death inducing signalling complex (DISC; Kischkel *et al* 1995; Figure 4.1). Induction of apoptosis via Fas can occur by two main pathways (Scaffidi *et al* 1998; Figure 4.1) and cells can be classified by means of the Fas induced apoptotic pathways used, all of which is determined by caspase kinetics. Caspase activation in Type I cells is rapid and mitochondria independent, whilst the delayed caspase activation of Type II cells is a mitochondria dependent mechanism.

Binding of Fas to its ligand, FasLigand (FasL/CD95L) results in the generation of a death-inducing signal in the Fas bearing cell. FasL is a 40kD type II membrane glycoprotein (Suda and Nagata, 1994), which is dependent upon ligation with its receptor, Fas in order to mediate Ca^{2+} independent cytotoxicity (Rouvier *et al* 1993).

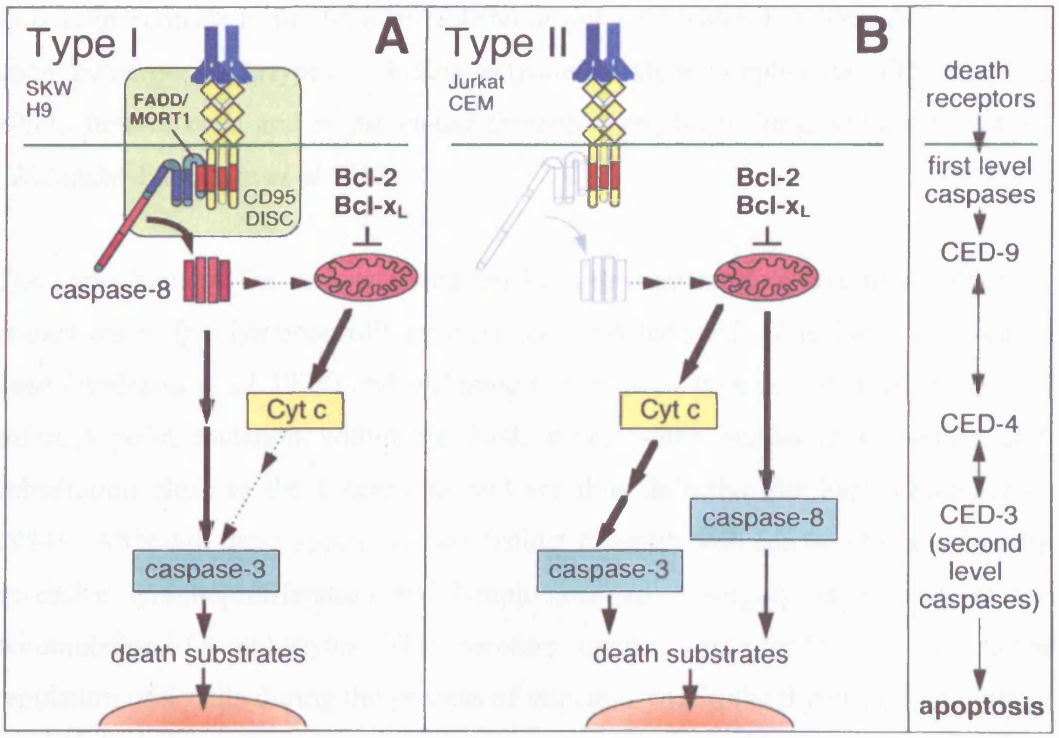

Figure 4.1 Model of the two CD95 signalling pathways.
(A) In type I cells CD95 triggering leads to strong caspase-8 activation at the DISC which bypasses mitochondria directly leading to activation of other caspases and subsequent apoptosis. (B) In type II cells a little DISC is formed leading to the activation of mitochondria, which results in caspase cleavage downstream of the mitochondria. Red box, death domain; blue box, death effector domain. Adapted from Scaffidi *et al* 1998.

Expression of FasL is known to be restricted to activated T cells (Anel *et al* 1994, Suda *et al* 1995, Vignaux *et al* 1995) and immune privileged sites (Streilein 1995). This is in contrast to the broader distribution of Fas, which has been demonstrated upon numerous cell types including activated mature lymphocytes (Trauth *et al* 1989), tumour cells and in the mouse thymus, liver, heart, lung, kidney and ovary (Watanabe-Fukunaga *et al* 1992).

The importance of Fas and its ligand has been demonstrated *in vivo* in spontaneous mutant mice. *lpr* (lymphoproliferation) mice are defective for Fas due to a truncated gene (Andrews *et al* 1978) and *gld* mice (generalised lymphoproliferative disease) suffer a point mutation within the FasL gene, which results in an amino acid substitution close to the C-terminus and are thus defective for FasL (Roths *et al* 1984). Although these appear as two distinct diseases both can be characterised by excessive lymphoproliferation and lymph node splenomegaly as a result of the accumulation of lymphocytes. This therefore supports a role for Fas and FasL in the regulation of T cells during the process of selection within the thymus (Kishimoto *et al* 1998) and a specific role in the induction of apoptosis during AICD or the deletion of peripheral T cells (Alderson *et al* 1995). Rat FasL cDNA was cloned first (Suda *et al* 1993) followed by that of human and mouse (Takahashi *et al* 1994) and all three have since been shown to share a high degree of amino acid homology (Figure 4.2). Mouse and rat FasL share a 91% amino acid homology while human and rat FasL share 84% homology at the amino acid level. FasL has been implicated in the maintenance of immune privilege, as demonstrated by the necessity of FasL for the control of invading lymphocytes, which have been shown to remain viable and exert local tissue damage in *gld* mice (Griffith *et al* 1995) where it was observed that FasL is located at areas of the BRB, on RPE cells and photoreceptor cells (Figure 4.2).

Expression of FasL has been documented at the cell surface and also in intracellular lysosomal type vesicles within epithelial cells (Ragnarsson *et al* 2000) and tumour cells (Hyer *et al* 2000) which is proposed to be a mechanism to prevent apoptosis. It

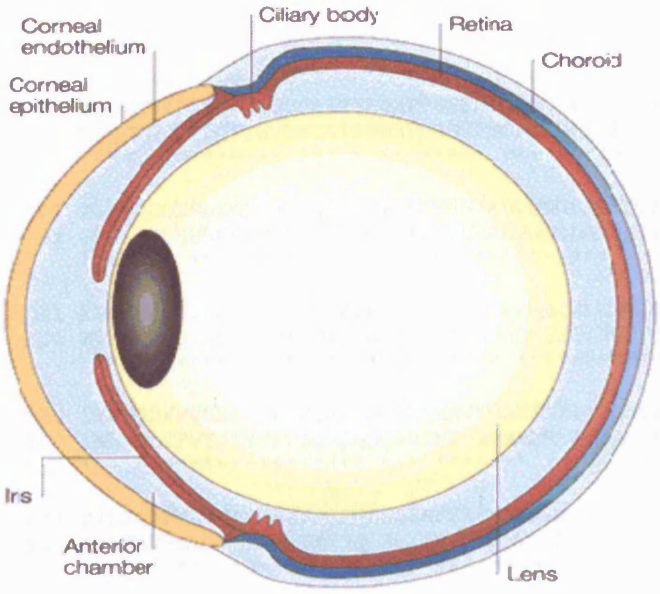

Figure 4.2 Localisation of FasL in the eye.
FasL is expressed in several strategic locations, including the cornea, retina, iris and ciliary body. FasL is located near areas that comprise the blood–ocular barrier and at locations where there is an opportunity for interaction between ocular tissue and inflammatory cells. In the retina, FasL is expressed on the retinal pigment epithelial cells, which comprise the outermost layer of the retina, and FasL is prominently expressed on the photoreceptors (rods and cones) where it might have a protective function in vision. FasL on the iris and ciliary body can contact and kill cells that enter from the vessels that are prominent in this tissue. In the cornea, FasL is expressed on the endothelium and epithelium, where it can control inflammatory cells that enter from the conjunctiva or anterior chamber. Taken from Green and Ferguson (2001)

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FASL_MOUSE      1 MQQPMNYPCPQIFWVDSSATSSWAPPGSVFPCPSCGPRGPDQRRPPPPPPPVSPLPPPSQ
FASL_RAT        1 MQQPVNYPCPQIYWVDSSATSPWAPPGSVFSCPSSGPRGPGQRRPPPPPPPPSPLPPPSQ
                  **** ******* ******** ******** *** ***** ********** ********

FASL_MOUSE     61 PLPLPPLTPLKKKDHNTNLWLPVVFFMVLVALVGMGLGMYQLFHLQKELAELREFTNQSL
FASL_RAT       61 PPPLPPLSPLKKKD-NIELWLPVIFFMVLVALVGMGLGMYQLFHLQKELAELREFTNHSL
                  * ***** ****** *  ***** ********************************** **

FASL_MOUSE    121 KVSSFEKQIANPSTPSEKKEPRSVAHLTGNPHSRSIPLEWEDTYGTALISGVKYKKGGLV
FASL_RAT      120 RVSSFEKQIANPSTPSETKKPRSVAHLTGNPRSRSIPLEWEDTYGTALISGVKYKKGGLV
                   **************** * *********** ****************************

FASL_MOUSE    181 INETGLYFVYSKVYFRGQSCNNQPLNHKVYMRNSKYPEDLVLMEEKRLNYCTTGQIWAHS
FASL_RAT      180 INEAGLYFVYSKVYFRGQSCNSQPLSHKVYMRNFKYPGDLVLMEEKKLNYCTTGQIWAHS
                  *** ***************** *** ******* *** ******** *************

FASL_MOUSE    241 SYLGAVFNLTSADHLYVNISQLSLINFEESKTFFGLYKL
FASL_RAT      240 SYLGAVFNLTVADHLYVNISQLSLINFEESKTFFGLYKL
                  ********** ****************************
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Figure 4.3: Alignment of rat and mouse FasL amino acid sequences showing 91% homology.

From: http://ca.expasy.org

has also been shown that $CD4^+$ T cells, $CD8^+$ T cells and natural killer cells contain intracellular stores of FasL which are released via secretory lysosomes (Bossi and Griffiths 1999). Cell surface FasL can also be cleaved to a 26kD soluble form termed soluble FasL (sFasL; Tanaka *et al* 1995, Kayagaki *et al* 1995). Human sFasL has been shown to induce apoptosis in Fas expressing cells whereas mouse sFasL is unable to induce apoptosis (Suda *et al* 1996). Production of sFasL is thought to be mediated by the action of matrix metalloproteinase-7 or matrilysin (Powell *et al* 1999, Mitsiades *et al* 2001, Vargo-Gogola *et al* 2002) and is proposed to be a downregulatory mechanism of FasL induced apoptosis (Suda *et al* 1997, Schneider *et al* 1998).

FasL is known to play a role in human disease as it is thought that tumour cells are able to evade the immune system by the loss of Fas and the induction of FasL expression (Strand *et al* 1996, Kim *et al* 2004). Elevated levels of sFasL are observed in ocular fluid during ocular inflammation (Sotozono *et al* 2000, Sugita *et al* 2000) and in tear fluid as a result of photorefractive keratectomy (Tuominen *et al* 1999), which suggests less membrane bound FasL. It is also extremely interesting, given the role of Fas and FasL in immune privilege within the eye (Griffith *et al* 1995), within the CNS (Streilein 1995, Bechmann *et al* 1999) and in the testis (Bellgrau *et al* 1995) that treatment of EAU with matrix metalloproteinase inhibitor BB-1101, an inhibitor of MMP-7, has been shown to prevent EAU, thus implicating the loss of membrane bound FasL in the pathogenesis of EAU (Wallace *et al* 1999). Fas and FasL have both been shown to contribute to the pathogenesis of EAE (Dittel 2000).

It has previously been shown that human macrovascular EC are able to induce apoptosis (Imanishi *et al* 2001) and that HUVECs express FasL (Walsh and Sata 1999) although expression at this site has been implicated in the facilitation of leukocyte extravasation (Walsh and Sata 1999). Expression of FasL by HUVECs has been shown to be downregulated in response to TNF-α (Walsh and Sata 1999). However IFN-γ has been shown to upregulate FasL upon Schwann cells *in vitro* and astrocytes upregulate FasL in response to IFN-γ and TNF-α (Choi *et al* 1999)

suggesting that the role for FasL may vary dependent upon the sites or tissues involved.

Aims

Having observed an increase in $CD4^+$ T cell apoptosis as a result of TEM across BEC monolayers, the aim of this chapter is to determine whether FasL is a candidate molecule for the induction of T cell apoptosis in this system. This will be investigated using a number of techniques in order to demonstrate FasL protein expression and functional FasL.

Results

Detection of FasL by immunocytochemistry

Initially experiments to investigate cell surface FasL expression by BEC were performed using a commercially available antibody and flow cytometry. Due to the reported low level expression of FasL upon cells not transfected to overexpress FasL we decided to use an indirect staining protocol using the biotinylated anti-rat FasL primary antibody MFL4 and amplification of any signal by the use of streptavidin-PE as a secondary antibody.

Given the reported effects of pro-inflammatory cytokines upon FasL expression in Schwann cells (Wohlleben *et al* 2000) and astrocytes (Choi *et al* 1999), in an attempt to maximise expression we investigated the effects of pro-inflammatory cytokines upon BEC FasL expression. Using the MFL4 antibody we investigated FasL expression by BEC at differing degrees of confluence, using increasing concentrations of antibody and increasing timeperiods in response to exposure to pro-inflammatory cytokines such as IFN-γ and TNF-α. However, we were unable to determine FasL expression under the conditions investigated (data not shown). We also investigated whether BEC contained intracellular stores of FasL, as a result of saponin-permeabilisation of cells, and the effects of pro-inflammatory cytokines upon levels of expression. However at each of the timepoints and conditions investigated, FasL expression by BEC was not observed above that of the non-specific binding of control, streptavidin-PE alone (data not shown). To determine whether our inability to confirm FasL expression was due to the detachment of the BEC from the culture flask FasL expression was investigated using confocal microscopy following a method previously described for staining of FasL upon T cells (Bossi and Griffiths 1999). However we were again unable to determine FasL expression above the levels of control (data not shown). Another possibility was that FasL expression by BEC was transient, we addressed this question by means of a matrix metalloproteinase inhibitor KB8301, (a kind gift of Professor Hideo Yagita of Juntendo University, Japan) which is an inhibitor of MMP-7 which is thought to play a role in the generation of sFasL. We therefore investigated the effects of KB8301 at varying

concentrations [0-20μM] upon cell surface and intracellular FasL, in unstimulated and cytokine stimulated BEC cultures. No FasL expression was detectable above that of control. Of interest during this study was that FasL expression by PMA and ionomycin stimulated T cells, a known positive control, was investigated in parallel, and under each of the conditions investigated no FasL expression was detectable.

As we were unable to determine FasL expression by any of the means investigated we set about to confirm using a different antibody or an alternative protocol, whether our inability to detect FasL was due to a lack of FasL expression by BEC or due to a limitation of the antibody employed in these studies. Given the close homology of rat FasL to human and mouse FasL we subsequently investigated a number of anti-human FasL antibodies in order to determine whether cross reactivity was observed by carrying out experiments as performed for the MFL4 antibody. Antibodies tested for this purpose included three anti-human FasL antibodies; 4H9 and 4A5, which were kind gifts of Beckman Coulter and an anti-human FasL antibody FSLO1 which was reported to exhibit cross reactivity with rat FasL as determined by western blotting. Staining of PMA and ionomycin and PHA stimulated human T cells resulted in FasL expression as determined using the 4A5 and 4H9 antibodies (data not shown). However, staining of PMA and ionomycin stimulated and Con A stimulated rat T cells with the 4A5 and 4H9 antibodies did not reveal FasL expression (data not shown). FSLO1, not previously tested for the purpose of flow cytometry failed to identify FasL expression upon human T cells using this method.

Soluble FasL was determined in BEC supernatants

In an attempt to confirm whether BEC express FasL, we set out to investigate whether sFasL was detectable in BEC culture supernatants as detectable using a commercially available mouse sFasL ELISA. sFasL was detectable in unstimulated and cytokine stimulated BEC cultures after 24h (Table 4.1) whilst levels in HEV cultures were found to below the sensitivity of the assay. Increasing concentrations

Supernatant	Soluble FasL (pg/ml)
BEC, Untreated	1563 ± 32.4
BEC, 100U/ml IFN-γ	1093 ± 226.0
BEC, 50U/ml IFN-γ	315 ± 44.8
BEC, 50U/ml IFN-γ + 5U TNF-α	942 ± 782.8
BEC, 50U/ml IFN-γ + 10U TNF-α	908 ± 1204.1
BEC, 50U/ml IFN-γ + 100U TNF-α	1322 ± 235.4
Activated T cell Control	1383 ± 304.1
Control supplied with kit	217 ± 40.9

Table 4.1 sFasL levels in BEC and T cell culture supernatants.
sFasL levels were detected in BEC culture supernatants in unstimulated and cytokine stimulated monolayers at 24h using a commercially available mouse FasL ELISA (R&D Systems). sFasL levels were detectable in each of the conditions investigated. Data is expressed as the means of duplicate wells ± S.D.

of IFN-γ resulted in a decrease in sFasL levels as compared to unstimulated controls whilst treatment of BEC monolayers with IFN-γ and increasing concentrations of TNF-α resulted in a dose dependent increase in sFasL levels.

FasL expression detectable in activated T cells by Western blotting

Given the promising results obtained by ELISA that BEC are capable of producing sFasL we investigated whether we could detect FasL using the anti-human FSLO1 antibody which was reported to detect FasL by Western blotting (see chapter 2, section 2.12). Western blot analysis of unstimulated and PMA/ionomycin stimulated rat T cells using the FSLO1 antibody resulted in the detection of a band of approximately 30kD, in the T cell lysate of PMA/ionomycin stimulated cells (Figure 4.4). Given that we were able to detect an activation dependent protein of approximately the predicted weight for FasL using an anti-human FasL antibody we would predict this to be FasL although subsequent protein analysis would be required in order to confirm this. Due to antibody and time constraints we were unable to investigate FasL expression upon BEC, although this data would suggest that this antibody may be suitable for the detection of rat FasL in BEC by western blotting.

BEC express FasL mRNA

In parallel to my studies, work within the laboratory carried out by Zoë Walters set about to investigate FasL expression by PCR. Some preliminary data has been generated which is extremely interesting and highly pertinent given our previous studies. Analysis of FasL using RT-PCR has shown that confluent BEC constitutively express FasL mRNA, however HEV express FasL mRNA at a much lower intensity (Figure 4.5A). The effects of the pro-inflammatory cytokines, IFN-γ and TNF-α upon FasL mRNA expression have also been studied. Despite an apparent increase in intensity of FasL mRNA an increase in β-actin was also detected and as a result semi-quantitative PCR analysis revealed that treatment of confluent monolayers with IFN-γ and TNF-α for 24h results in a downregulation of FasL mRNA relative to untreated BEC monolayers (Figure 4.5B). IFN-γ would appear to be more effective in the downregulation of FasL mRNA expression than TNF-α,

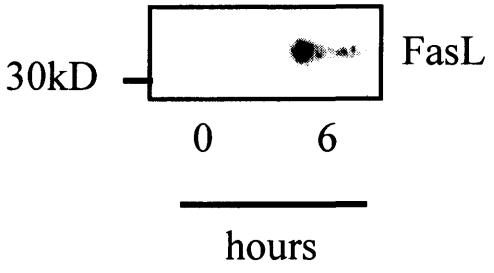

Figure 4.4 Western blot analysis of FasL in unstimulated and stimulated T cells. Western blot analysis of unstimulated and PMA and ionomycin stimulated rat T cells using the FSLO1 antibody revealed a protein band of approximately 30kD.

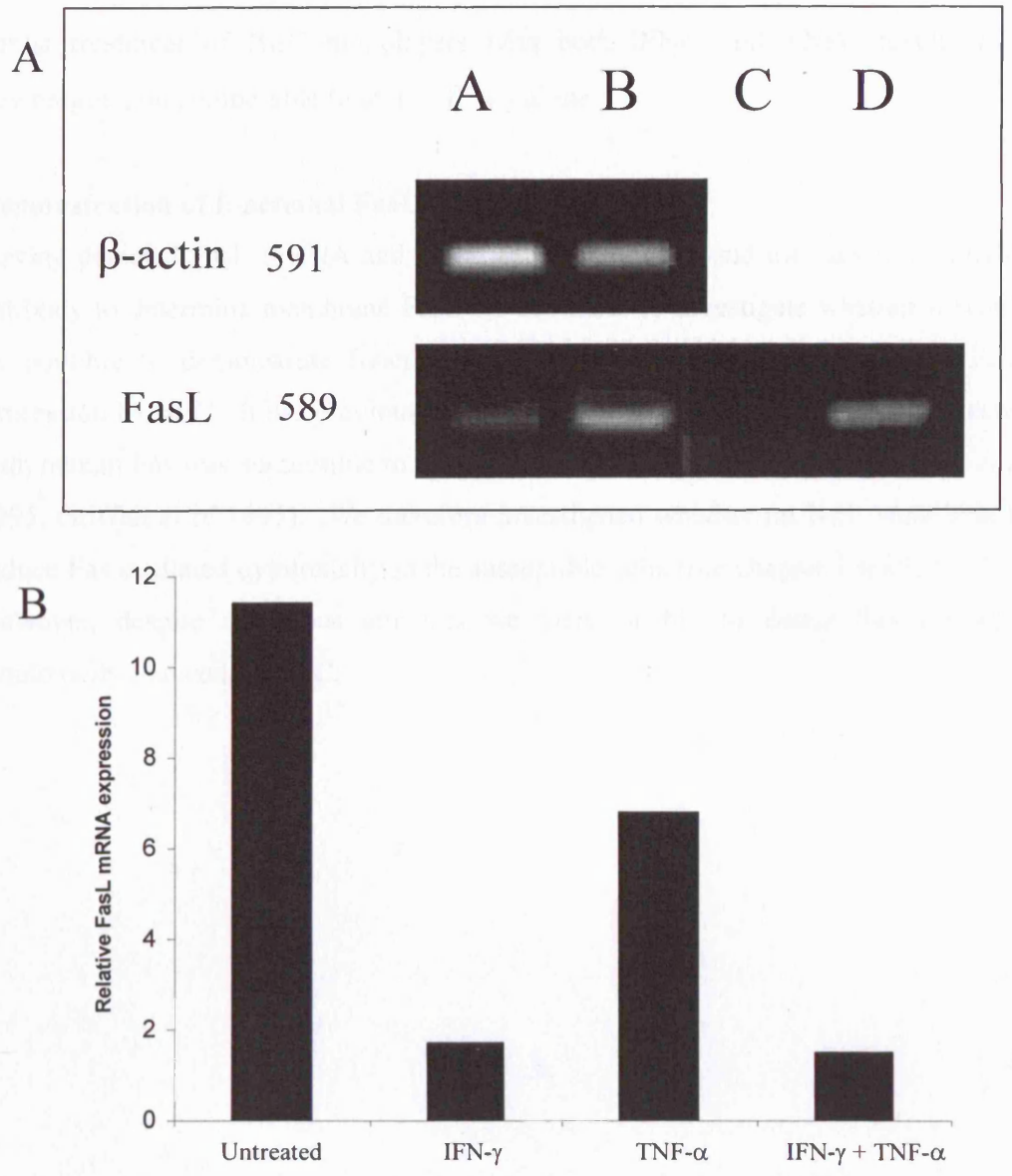

Figure 4.5 FasL mRNA analysis of confluent unstimulated BEC and HEV monolayers and expression in response to IFN-γ and TNF-α.
Panel A shows FasL mRNA expression by unstimulated BEC and HEV monolayers. Lane A: HEV; lane B: BEC; lane C: negative control and lane D: T cell control. Semi-quantitative PCR analysis revealed a low level expression of FasL mRNA in HEV monolayers as compared with a band of high intensity in BEC monolayers. Panel B shows quantification of FasL relative band intensities from BEC monolayers stimulated with IFN-γ [50U/ml], TNF-α [100U/ml] or with IFN-γ [50U/ml] and TNF-α [100U/ml] for 24h. All positive bands were of the expected size. Relative band intensity was then measured using ScanAnalysis v2.50. The relative amounts of FasL mRNA expression were calculated as a proportion of the β-actin expression in each sample.

whilst treatment of BEC monolayers with both IFN-γ and TNFα results in a downregulation comparable to that of IFN-γ alone.

Demonstration of functional FasL

Having detected FasL mRNA and sFasL in BEC cultures and the lack of a suitable antibody to determine membrane FasL we set about to investigate whether it would be possible to demonstrate functional FasL so as to indirectly determine FasL expression by BEC. It has previously been reported that a mouse cell line transfected with human Fas was susceptible to apoptosis mediated by mouse FasL (Brunner *et al* 1995, Griffith *et al* 1995). We therefore investigated whether rat BEC were able to induce Fas mediated cytotoxicity in the susceptible cells (see chapter 2 section 2.8.5). However, despite numerous attempts we were unable to detect Fas mediated cytotoxicity induced by BEC.

Discussion

Our previous findings demonstrate that BEC but not HEV cells induce T cell apoptosis in $CD4^+$ T cells, following TEM across an *in vitro* model of the BBB (Chapter 3). It is recognized that EC forming the BBB are specialised for this function. The CNS is known to be an immune privilege site and FasL has previously been reported to play a role in the induction of apoptosis as a mechanism of immune privilege within the eye (Griffith *et al* 1995). We therefore hypothesized that the induction of apoptosis by BEC may be an additional specialized property of BEC of the BBB and therefore decided to investigate whether FasL may be a candidate for this role in our model system.

Initially experiments set out to determine cellular expression of FasL by BEC. However, using a panel of antibodies, we were unable to determine FasL expression at the cell surface, intracellularly, in the presence of a matrix metalloproteinase inhibitor or in response to pro-inflammatory cytokines. It has previously been reported that FasL expression may be transient or relatively low in cells, which are not induced to overexpress FasL (BD Bioscience). Given that we were able to determine FasL expression upon human T cells using anti-human FasL antibodies, but not upon rat T cells would suggest a low degree of species cross reactivity of the antibody, despite a high degree of homology between human and rat FasL at the amino acid level. However FasL expression has been documented in the normal rat brain by means of immunohistochemical techniques (Bechmann *et al* 1999). Therefore it would be intriguing to investigate whether antibodies for this purpose may be able to detect FasL upon BEC.

Despite our inability to determine FasL expression within the cell we were able to determine sFasL in BEC culture supernatants and levels of sFasL were modulated in response to pro-inflammatory cytokines. This data indicates that BEC are capable of synthesising and expressing FasL, even if only transiently. IFN-γ appears to down regulate FasL production, and increasing concentrations of TNF-α in the presence of

IFN-γ appear to inhibit IFN-γ induced downregulation of sFasL production. Also of interest was that sFasL in culture supernatant harvested from HEV monolayers was below the sensitivity of detection. This data is consistent with that presented in Chapter 3 whereby BEC but not HEV monolayers are able to induce T cell apoptosis. Therefore it is a possibility that the induction of apoptosis is a specialised function of BEC. This data also provides an insight into the functional nature of rat sFasL. Given that sFasL was detectable in culture supernatant after 24h we would expect BEC to secrete sFasL during the course of our co-culture assays (up to 72h), however no significant increase in T cell apoptosis was detectable at each of the timepoints investigated in our non-contact assay (see chapter 3), thus it would suggest that rat BEC-derived sFasL is biologically inactive or that it may act to prevent T cell apoptosis. This data complements that of the functional activity of murine sFasL (Suda *et al* 1996) and as such may be considered as a mechanism of FasL downregulation (Suda *et al* 1997). Human EC have been shown to produce sFasL in response to hypoxia (Mogi *et al* 2001), this data therefore supports a role for rat BEC and rat T cells in the production of sFasL.

We therefore decided to investigate FasL protein expression by Western blotting and can report that rat T cells stimulated with PMA and ionomycin for 6h express a T cell activation dependent protein of the approximate molecular weight using the FSLO1 anti-human FasL antibody. This data is in keeping with the reported expression of FasL upon activated T cells (Anel *et al* 1994, Suda *et al* 1995, Vignaux *et al* 1995). Unfortunately our present studies were unable to extend to determine FasL expression upon BEC by Western blotting. However, this promising data would suggest that this antibody may be suitable for the detection of FasL in BEC. This data, unlike that of the flow cytometry studies, supports a role for inter-species cross reactivity. This is most likely due to the different antibody employed or as a result of conformational changes to rat FasL which may make it more amenable to recognition by an anti-human FasL antibody.

Given our conflicting findings, studies were undertaken in order to investigate FasL mRNA expression by RT-PCR. Preliminary results suggest that BEC constitutively express FasL to a greater level than HEV monolayers and semi-quantitative PCR analysis also revealed that IFN-γ and TNF-α down regulated FasL mRNA expression as compared to unstimulated cells. This is in contrast to the role of IFN-γ in the upregulation of FasL upon Schwann cells of the peripheral nervous system (PNS; Wohlleben *et al* 2002) and upregulation of FasL upon human astrocytes in response to both IFN-γ and TNF-α (Choi *et al* 1999). Our data is consistent with the effects of TNF-α upon a non CNS vascular endothelium (Sata and Walsh 1998, Walsh and Sata 1999) suggesting that BEC are capable of FasL gene transcription. In order to conclusively state that BEC express FasL, confirmation of protein expression of FasL is necessary. In light of the PCR data it would suggest that in our attempts to maximize FasL expression by treatment with cytokines we were, in fact, inhibiting FasL expression. It would therefore be extremely interesting to reinvestigate FasL protein expression by BEC in the absence of IFN-γ and TNF-α and possibly in the presence of interferon-α which has been shown to upregulate FasL mRNA expression upon peripheral blood mononuclear cells (Kaser *et al* 1999).

Our data supports the hypothesis that BEC are capable of expressing FasL, despite our inability to confirm cell surface expression, which we feel may be due to the lack of a reliable antibody. However, as we have shown in chapter 3, EC induced T cell apoptosis is cell-contact dependent. This therefore supports a role for a cell surface molecule. It is also a possibility that the apoptosis observed is not due to FasL, and that a role for an alternative cell surface mediator could exist. It would also be interesting to investigate whether it would be possible to demonstrate the presence of functional FasL using our *in vitro* transmigration system. Given the PCR data demonstrating IFN-γ downmodulation of FasL mRNA, experiments to investigate whether pre-treatment of BEC monolayers with IFN-γ prior to assay are currently being carried out, to assess whether an inhibition of $CD4^+$ T cell apoptosis is observed. In addition the inhibition of FasL expression by treatment with vitamin E is also being investigated (Li-Weber *et al* 2002). Vitamin E has been reported to

inhibit FasL mRNA expression in other systems and it will be interesting to investigate whether the effects observed at the gene level are evident at the protein level.

Chapter 5

Immunomodulation by statins *in vitro*

Introduction

The ICAM-1–LFA-1 interaction is known to play a major role in the multistep process of leukocyte migration at the vascular endothelium. EC ICAM-1 has further been shown to play an essential role in leukocyte extravasation within the CNS (Greenwood *et al* 1995, Pryce *et al* 1997, Reiss *et al* 1998) and also in the peripheral vascular system (Oppenheimer-Marks *et al* 1991, Shimizu *et al* 1992).

ICAM-1 has previously been shown to interact with a number of intracellular proteins including α-actinin, the actin binding protein and the microtubule associated β-tubulin (Federici *et al* 1996) thus suggesting an association of ICAM-1 with the EC cytoskeleton (Amos *et al* 2001). It is proposed that ICAM-1 forms a homodimer (Casasnovas *et al* 1998) upon which an EC signalling molecule is generated (Hubbard and Rothlein 2000). ICAM-1 cross linking mediated by antibody or lymphocyte adhesion has shown to result in the tyrosine phosphorylation of a number of cytoskeletal proteins including focal adhesion kinase (FAK), paxillin and $p130^{Cas}$ (Etienne *et al* 1998) in addition to the induction of cytoskeletal arrangements (Adamson *et al* 1999), calcium signalling (Clayton *et al* 1998) and the regulation of TJ in epithelia (Nusrat *et al* 1995). The role for ICAM-1 in actin cytoskeleton reorganisation is supported by the inhibition of lymphocyte transmigration as a result of cytochalasin D treatment (Adamson *et al* 1999). *In vitro* studies have revealed that intracellular ICAM-1 (Amos *et al* 2001, Lyck *et al* 2003, Greenwood *et al* 2003b) and ICAM-1 mediated cytoskeletal modifications are associated with increased levels of activated Rho and that lymphocyte migration across BEC is a Rho-dependent pathway (Adamson *et al* 1999).

Rho proteins are a family of small guanosine triphosphate (GTP) binding proteins of the Ras superfamily and are so-called because of their homology with Ras (Ras homologous). This family of 20-30kD monomeric proteins are thought to act as molecular switches to regulate numerous cellular processes, by cycling through an active GTP bound state and an inactive GDP bound state (Mackay and Hall 1998).

These proteins have previously been described to play a role in cytoskeleton organisation (Paterson *et al* 1990, Ridley and Hall 1992), gene transcription (Coso *et al* 1995, Minden *et al* 1995), phagocytosis (Caron and Hall 1998) and pinocytosis (Ridley 1997). The most interesting role of which, given our present study, includes the role of Rho proteins in cell migration (Nobes and Hall 1999). These proteins are also thought to regulate tight junctions (Hall 1998) and work within the laboratory has shown that Rho proteins play a role in the regulation of lymphocyte TEM via ICAM-1 signalling (Adamson *et al* 1999).

Rho proteins cycle between an active GTP bound state and an inactive guanosine diphosphate (GDP) bound state (Boguski and McCormick 1993; Figure 5.1). This activation cycle is regulated by three main means; guanine nucleotide dissociation inhibitors (GDI's), guanine nucleotide exchange factors (GEF's) and GTPase-activation proteins (GAP's). GDI's are thought to bind to inactive RhoGDP proteins and result in their stabilisation. Dissociation of the GDI is thought to allow nucleotide exchange and activation mediated by GEFs. GAPs are responsible for the hydrolysis of Rho GTP to RhoGDP, which is subsequently sequestered by Rho-GDI. Active RhoGTPases are thought to interact with 30 or more potential effector proteins (reviewed by Bishop and Hall 2000) in order to initiate coordinated cellular responses.

A number of proteins of the Ras superfamily including the RhoGTPases contain a carboxyl terminal CAAX sequence, where C represents a cysteine, A represents an aliphatic amino acid and X represents any amino acid. This amino acid sequence is known to be a signal for three types of post-translational modification including prenylation, proteolysis and carboxyl group methylation (Hancock *et al* 1991). It has since been reported that Rho proteins undergo prenylation (Adamson *et al* 1992) which occurs via the C-terminal addition of C_{15} (farnesyl; Casey *et al* 1989; Hancock *et al* 1989) or C_{20} (geranylgeranyl; Epstein *et al* 1990, Farnsworth *et al* 1990, Rilling *et al* 1990) isoprenoid structures. RhoA and RhoC are thought to undergo geranyl-

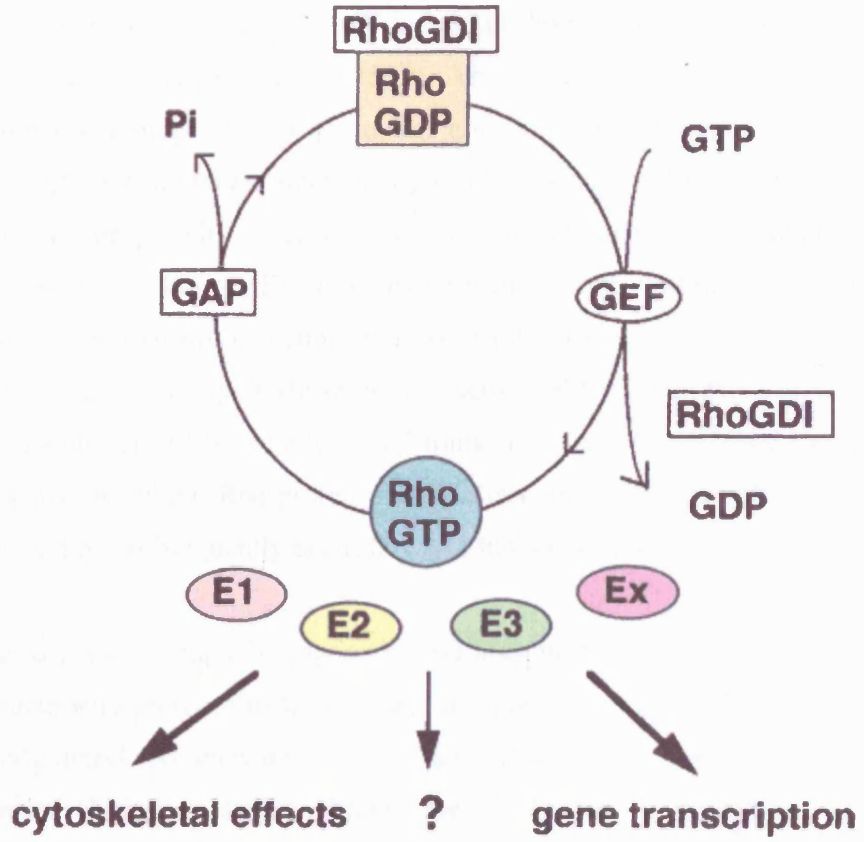

Figure 5.1 The Rho GTPase cycle.
Rho GTPases are thought to exist in an inactive GDP-bound state complexed to one of the three known Rho-GDIs. In response to an incoming signal such as bradykinin, Rho-GDI dissociates, and a GEF catalyses nucleotide exchange and activation. It is thought that this is likely to occur at or close to the plasma membrane, because it is unlikely that isoprenylated GTPases will exist free in the cytosol. In its active state, the GTPase interacts with cellular targets or effectors (*E1-x*) to generate a cellular response. Currently around eight potential targets are known for each of the three GTPases, Rho, Rac, and Cdc42, but there is no reason to believe that this list is complete. Most of the targets are ubiquitously expressed, and it is unknown how a GTPase "chooses" its target under a particular set of circumstances. Finally, one of around 10 known Rho GAPs will interact with the GTPase-target complex and catalyse GTP hydrolysis. The GDP-bound form of the GTPase is then "extracted" from the membrane by Rho-GDI, and the cycle is complete. The exact role of Rho-GDI is unclear, and the rate-determining step in the cycle has not been determined. What is clear, however, is that Rho GTPases can, in their GTP-bound state, interact with numerous target proteins to induce coordinated signals. Taken from Mackay and Hall 1998.

geranyl prenylation while RhoB exists in both a farnesyl and geranylgeranyl prenylated form (Adamson *et al* 1992). The process of protein prenylation is known to influence biological activity, in that non-prenylated Rho proteins localize to the cytosol of the cell and are functionally inactive, while post-translational modification and activation permits interaction with cell membranes (Philips *et al* 1993, Boivin and Beliveau 1995). Previous experiments from within the laboratory have demonstrated that myristolation of Rho results in the generation of functional Rho and thus permits lymphocyte migration across BEC (Greenwood *et al* 2003). One known inhibitor of Rho function is C3 transferase from *Clostridium botulinum,* which was shown to inhibit Rho protein function through ADP ribosylation (Aktories *et al* 1989) and has subsequently been shown to inhibit lymphocyte TEM.

More recently a group of compounds have been identified which are able to indirectly modulate Rho protein function. These compounds act as inhibitors of 3-hydroxy-3-methylglutaryl co enzyme A reductase (HMG CoA reductase) and are more commonly known as statins. Statins are widely used in the clinical setting for the treatment of hypercholesterolemia (The Scandinavian Simvastatin Survival Study Group, 1994 and Shepard *et al* 1995). HMG-CoA reductase is the enzyme which regulates the rate-limiting step of cholesterol synthesis.

Statins function to inhibit the conversion of 3-hydroxy-3-methylglutaryl Co enzyme A (HMG CoA) to mevalonate through the inhibition of HMG CoA reductase function. Inhibition of HMG CoA reductase results in the inhibition of cholesterol synthesis. Figure 5.2 is a schematic diagram of the cholesterol synthesis pathway in mammals. In addition to this, inhibition of mevalonate production results in the inhibition of subsequent intermediates of the cholesterol synthesis pathway which act as donors of isoprenoid pyrophosphate substrates such as the 15-carbon farnesyl or 20-carbon geranylgeranyl pyrophosphates (FPP, GGPP) which are essential for a number of cellular processes. Depletion of mevalonate by HMG-CoA reductase inhibition has been shown to result in the accumulation of unmodified proteins as a result of limited FPP and GGPP availability (Schafer *et al* 1989, Leonard *et al* 1990).

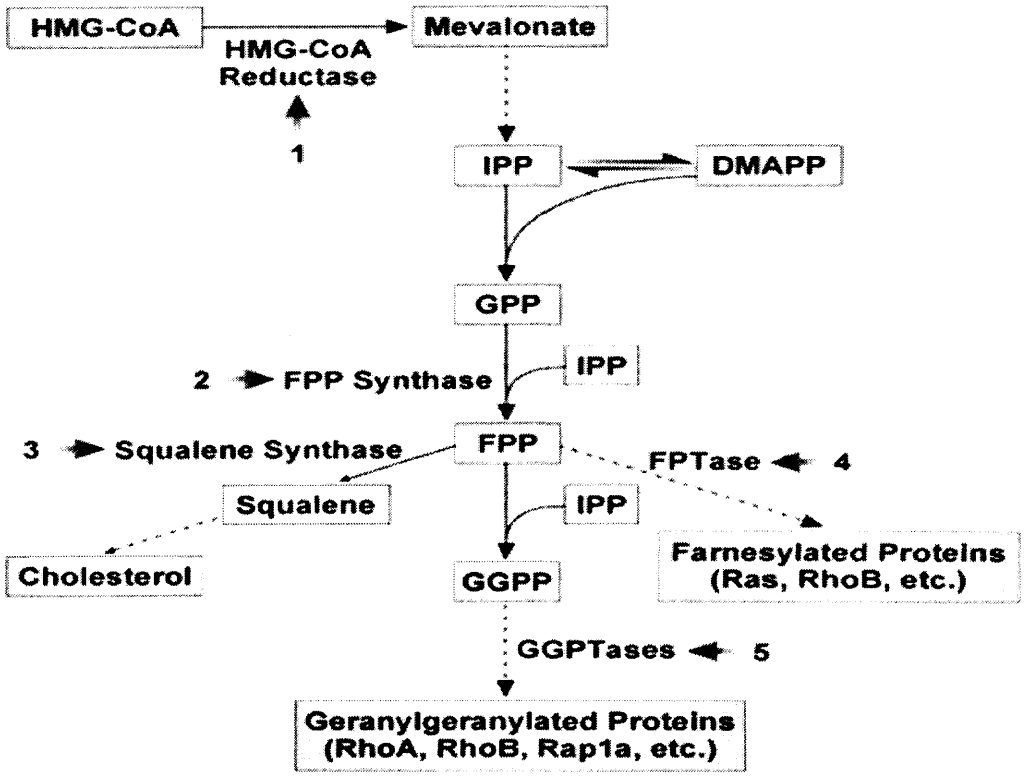

Figure 5.2 The Isoprenoid biosynthetic pathway.
Arrows indicate the site of action targeted by a number of inhibitors of the isoprenoid biosynthetic pathway. The action of HMG-CoA reductase inhibitors or statins is represented as target 1. Adapted from Holstein *et al* 2002.

Isoprenoid pyrophosphate substrates are essential for the generation of functional Rho proteins (Zhang and Casey 1996). Statins, through the inhibition of isoprenoid donating compounds therefore inhibit the generation of functional Rho. Since functional Rho is essential for ICAM-1 mediated TEM across CNS derived monolayers (Adamson *et al* 1999), statins are likely to inhibit this process.

It has previously been demonstrated that protein prenyl transferase inhibitors prevent TEM as *in vitro* using BEC and also *in vivo* in EAE (Walters *et al* 2002) thus supporting the requirement of functional Rho for lymphocyte migration. Statins have previously been demonstrated to inhibit TEM *in vitro* using BEC (Greenwood *et al* 2003) and also lymphocyte traffic *in vivo* in EAE (Youssef *et al* 2002, Greenwood *et al* 2003). Given that the BBB and BRB share numerous properties it was decided to investigate whether statins may influence TEM in an *in vitro* model of the BRB using REC.

Aims

The previous chapters document a role for CNS EC in the modulation of T lymphocyte function as a result of TEM. In this chapter we aim to investigate whether statins are able to modulate the effects of CNS EC upon T lymphocyte function.

More specifically this chapter aims to investigate the effects of lovastatin upon lymphocyte adhesion to and migration across REC and RPE monolayers as *in vitro* models of the BRB and to investigate the role of ICAM-1 and its intracellular signalling pathway in this process.

Results

Lovastatin affects T cell viability in a time- and dose-dependent manner

An initial study was conducted to investigate the effects of increasing concentrations of lovastatin on T cell function, in particular T cell viability. Viability was determined by PI exclusion. T cells were either untreated or incubated with increasing concentrations of lovastatin [1-100μM] for up to 120h (Table 5.1). All incubations were carried out in the presence of IL-2. Results are expressed as the means of three independent experiments ± SD calculated as a percentage of the positive control without lovastatin.

Lovastatin suppressed T cell viability in a dose- and time-dependent manner. Of importance for the purposes of our immediate study is that at 4h (the maximum exposure of T cells to lovastatin for the duration of the migration assay) lovastatin showed no significant effect upon T cell viability except upon T cells cultured in the presence of 100μM lovastatin ($p \leq 0.05$; Table 5.1).

Lovastatin does not influence REC viability

Previous results from within the laboratory have determined optimal depletion of functional Rho within BEC to occur as a result of treatment of EC monolayers with C3 transferase for 24h (Adamson *et al* 1992). We therefore investigated the effects of increasing concentrations of lovastatin upon EC viability at this time. REC monolayers as an *in vitro* model of the BRB were pretreated with increasing concentrations of lovastatin [0-100μM] and also C3 transferase [10μg/ml] for 24h and cell viability determined by PI exclusion. Lovastatin showed no significant effect upon EC viability at 24h at each of the concentrations investigated. C3 transferase elicited no significant effect upon EC viability at 24h (Figure 5.3) as compared to untreated controls.

	[Lovastatin] μM					
Hours (h)	0	0.1	1	10	50	100
1	100.0 ± 0.0	100.2 ± 3.7	100.5 ± 0.9	99.6 ± 0.6	93.0 ± 3.4*	74.9 ± 22.9
2	107.3 ± 6.5	105.4 ± 7.6	107.6 ± 8.1	107.7 ± 4.1†	97.8 ± 11.4	72.5 ± 27.4
4	105.0 ± 7.0	104.3 ± 10.6	106.8 ± 4.6	103.9 ± 6.1	96.1 ± 11.2	42.1 ± 25.1*
6	100.1 ± 10.2	100.0 ± 10.0	99.2 ± 5.9	97.3 ± 3.8	92.0 ± 15.6	27.7 ± 22.0**
24	95.2 ± 21.0	90.5 ± 14.9	89.1 ± 15.4	74.6 ± 7.7	52.5 ± 4.9*	5.8 ± 1.7*††
48	89.5 ± 33.2	88.0 ± 38.2	64.4 ± 21.0†	5.4 ± 4.0*††	5.4 ± 6.6*††	0.0 *††
120	57.0 ± 31.1	51.5 ± 33.2	11.2 ± 8.3††	0.1 ± 0.2*††	0.1 ± 0.1**††	0.0*††

Table 5.1 Lovastatin exerts a time- and dose-dependent effect upon T cell viability *in vitro*.
Table shows the mean percentage viability of Con A line T cells treated with increasing concentrations of lovastatin [0-100μM] for increasing periods as a percentage of control untreated cells. Cell viability was assessed by PI exclusion and flow cytometry. Values are expressed as a percentage of control viability. Values represent the means of three independent experiments ± S.D. Statistical analysis was performed using the Students T Test. * $p \leq 0.05$ compared with untreated control of same concentration, ** $p \leq 0.01$ compared with untreated control of same concentration, † $p \leq 0.05$ as compared with respective 1h timepoint of control, †† $p \leq 0.01$ as compared with respective 1h timepoint of control.

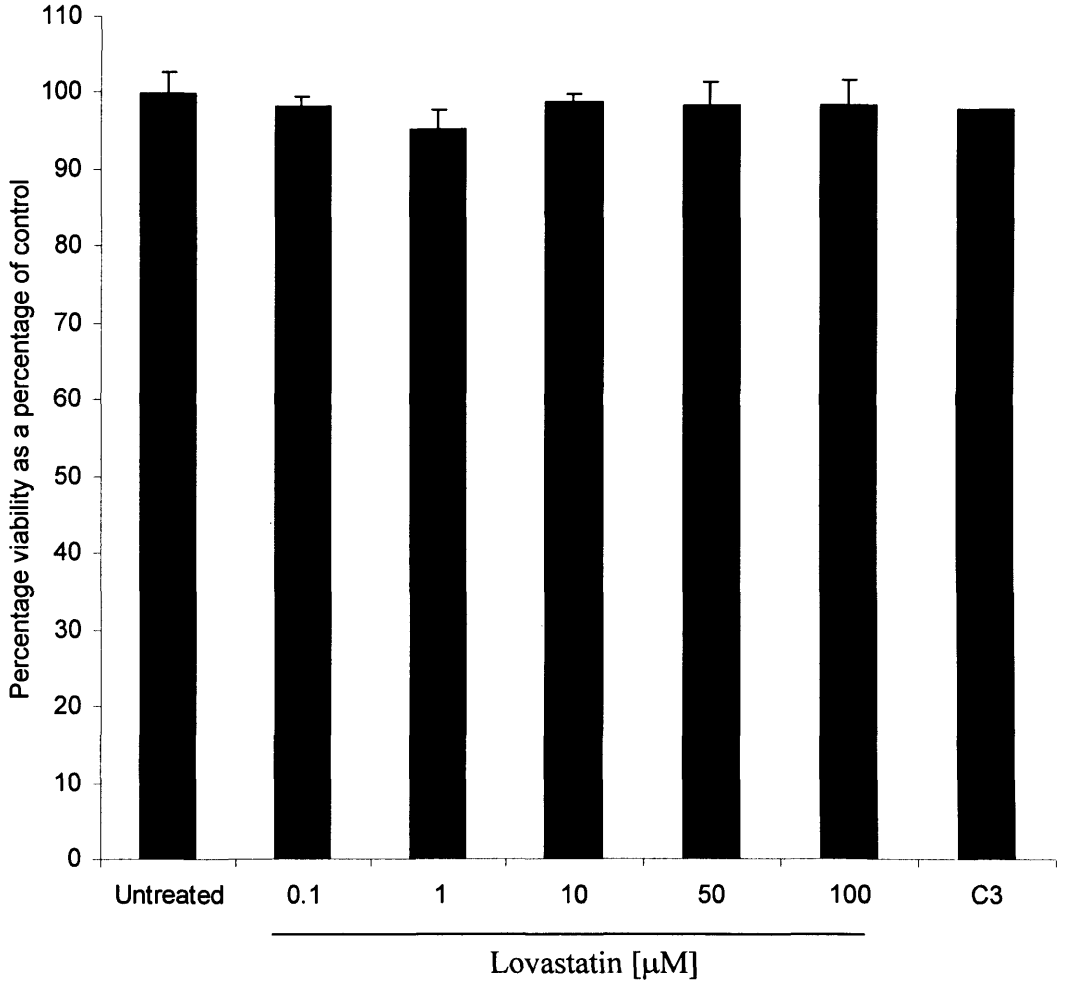

Figure 5.3 Increasing concentrations of lovastatin for 24h does not affect REC viability.

Percentage viability of REC populations was determined in populations pretreated for 24h with increasing concentrations of lovastatin [0-100μM] or 16h with C3 transferase [10μg/ml]. Cell viability was determined by PI exclusion and flow cytometry. Values are expressed as a percentage of untreated control viability. Bars represent the means of three independent experiments ±S.D. Statistical analysis was performed using the Students T Test.

Treatment with lovastatin for 4h does not affect lymphocyte adhesion or migration

Subsequent experiments set out to determine the effect of lovastatin treatment on PLN adhesion to and antigen-specific T cell migration across REC monolayers. Mean basal migration of antigen-specific T cells across untreated monolayers was found to be 36.8 ± 6.8% while mean basal adhesion to REC monolayers was found to be 13.6 ± 2.6% (data not shown).

Previous studies determined that pretreatment of EC for 4h with statins has no effect upon T cell adhesion or migration using an *in vitro* model of the BBB (Greenwood *et al* 2003). Initially we investigated the effects of REC exposure to lovastatin for 4h. No significant difference in the level of PLN adhesion or antigen-specific T cell migration was detectable between lovastatin-treated and untreated monolayers (Figure 5.4).

Pre-treatment of REC with lovastatin for 24h inhibits lymphocyte migration

Given our previous findings that exposure of BEC monolayers to lovastatin for 24h has been shown to result in the depletion of intracellular stores of isoprenyl groups thereby inhibiting TEM whilst having no detectable effect upon leukocyte adhesion (Greenwood *et al* 2003), we decided to investigate the effects of increasing concentrations of lovastatin on PLN adhesion and antigen-specific T cell migration upon REC after 24h pre-treatment (Figure 5.5). Small but significant decreases in PLN adhesion to lovastatin treated monolayers were detectable in a dose dependent manner in cultures pretreated with lovastatin [1-100μM] (100μM; 83.4 ± 1.9% as compared to untreated control; 100 ± 1.8%; $p \leq 0.05$ and $p \leq 0.001$) whilst a small but significant increase in PLN adhesion to C3 transferase treated monolayers was observed as compared to untreated controls (110.9 ± 3.9%; $p \leq 0.01$; Figure 5.5). In contrast pretreatment with increasing concentrations of lovastatin resulted in highly significant decreases in the level of TEM at all concentrations investigated ($p \leq 0.001$). Using a known inhibitor of TEM, levels of lymphocyte migration across C3 transferase-treated monolayers were comparable with that observed for REC monolayers pretreated with 1μM lovastatin.

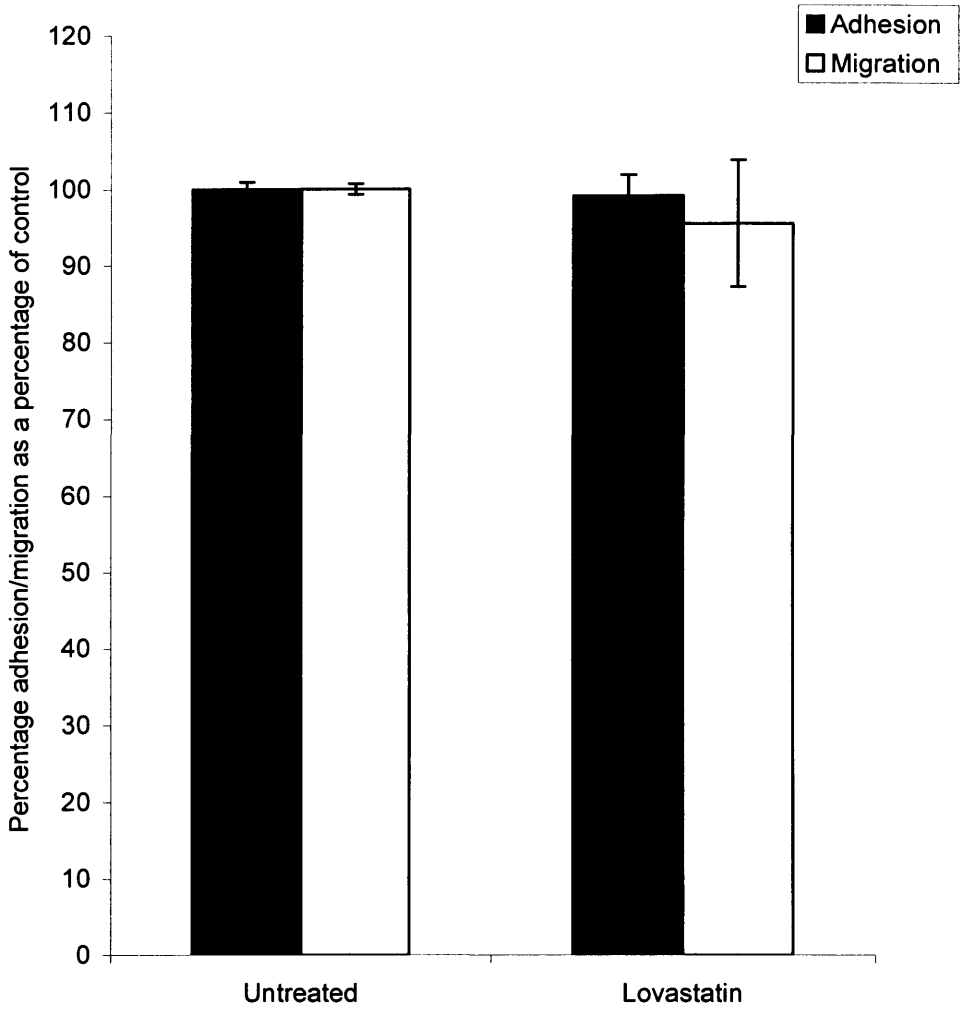

Figure 5.4 Exposure of REC and T cells to 10μM lovastatin for 4h showed no significant effect upon T cell migration or adhesion to REC.
REC were cultured in the presence of 10μM prior to and during the assay for a maximum of 4h. Means are expressed as a percentage of control adhesion or migration. Ten (migration) or twelve (adhesion) wells were assayed per condition per experiment. Each experiment was performed in triplicate. Results are expressed as the means ± SEM. Statistical analysis was performed using the Student's T-test.

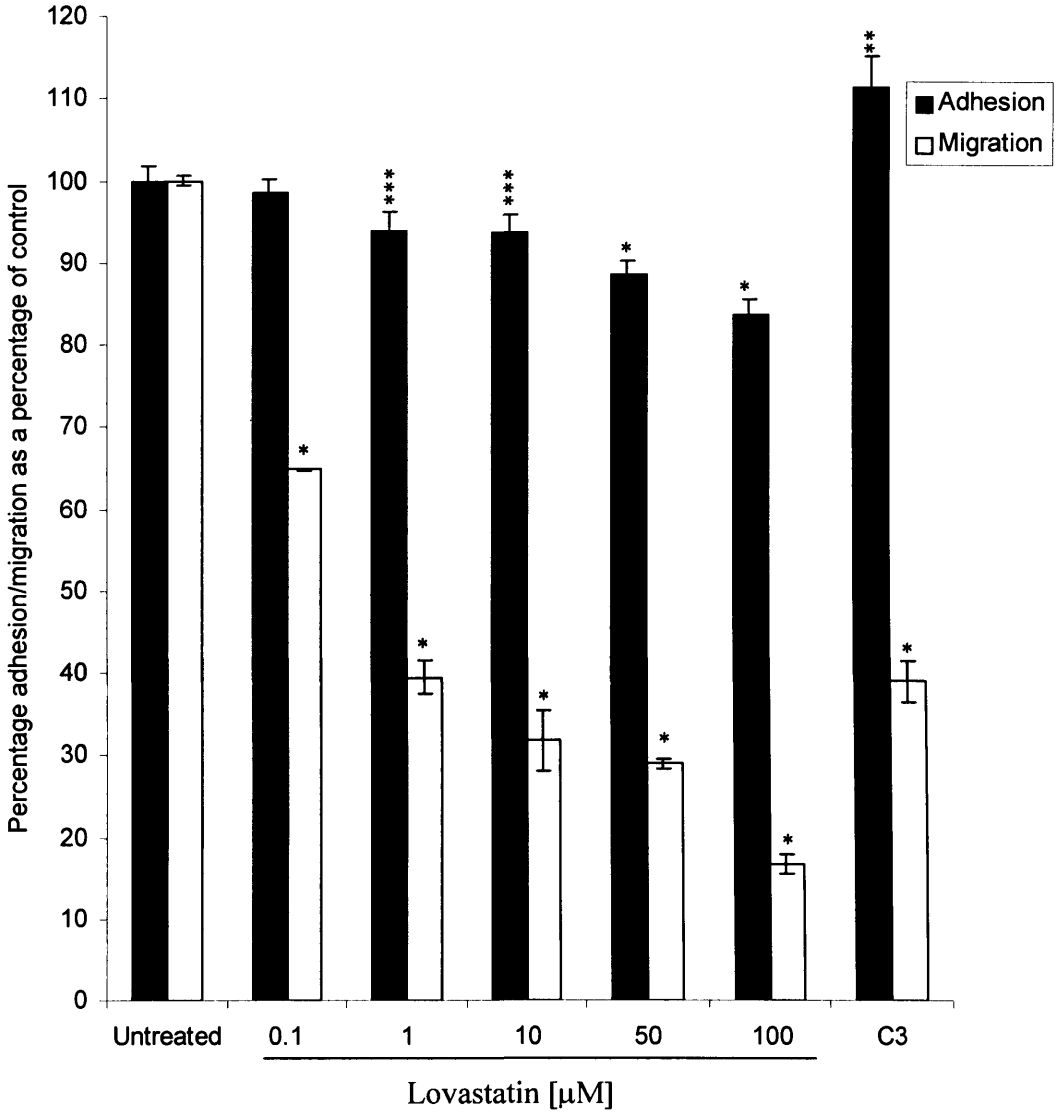

Figure 5.5 pretreatment of REC with lovastatin results in a significant inhibition of lymphocyte migration and a small but significant effect upon T cell adhesion. REC monolayers were pre-treated with increasing concentrations of lovastatin [0-100μM] for 24h or 16h with C3 transferase [10μg/ml]. Means are expressed as a percentage of control adhesion or migration. Ten (migration) or twelve (adhesion) wells were assayed per condition per experiment. Each experiment was performed in triplicate. Results are expressed as the means ± SEM. Statistical analysis was performed using the Student's T-test. *$p < 0.001$, **$p < 0.01$, ***$p < 0.05$ compared with untreated control.

Lovastatin treatment does not affect ICAM-1 cell surface expression

To establish whether lovastatin altered REC expression of ICAM-1 and therefore REC capacity for T cell adhesion and migration we set about to determine REC ICAM-1 expression in response to increasing concentrations of lovastatin. For this purpose REC monolayers were again pretreated with lovastatin [0-100μM] and the level of cell surface ICAM-1 expression determined by flow cytometry. Our data suggests that lovastatin does not affect REC cell surface expression of ICAM-1 (Figure 5.6).

Supplementation with mevalonolactone but not squalene restores lymphocyte TEM across lovastatin treated REC

Given our hypothesis that statins disrupt the ICAM-1 signalling pathway by the specific inactivation of Rho proteins, we set about to demonstrate that lovastatin inhibited migration as a result of the inhibition of isoprenoid pyrophosphate intermediates, which are essential for the generation of functional Rho and not as a result of the inhibition of the cholesterol synthesis pathway. Experiments were carried out in order to determine whether supplementation of lovastatin treated monolayers with exogenous squalene, a precursor of cholesterol, or mevalonolactone, a precursor of isoprenoid donating compounds, could rescue the inhibitory effects of lovastatin. Given our hypothesis, we would predict that addition of exogenous mevalonolactone but not squalene, could restore lymphocyte TEM. In order to investigate this, REC monolayers were either untreated or pretreated with lovastatin [10μM], mevalonolactone [5mM] or squalene [10μM] alone or with lovastatin in the presence of mevalonolactone or squalene (Figure 5.7).

Pretreatment with lovastatin or lovastatin and squalene resulted in a small but significant decrease in PLN adhesion; 93.5 ± 2.1% ($p \leq 0.05$) and 91.5 ± 2.6% ($p \leq 0.05$) respectively, as compared to control (100 ± 2.1%) while adhesion of PLN

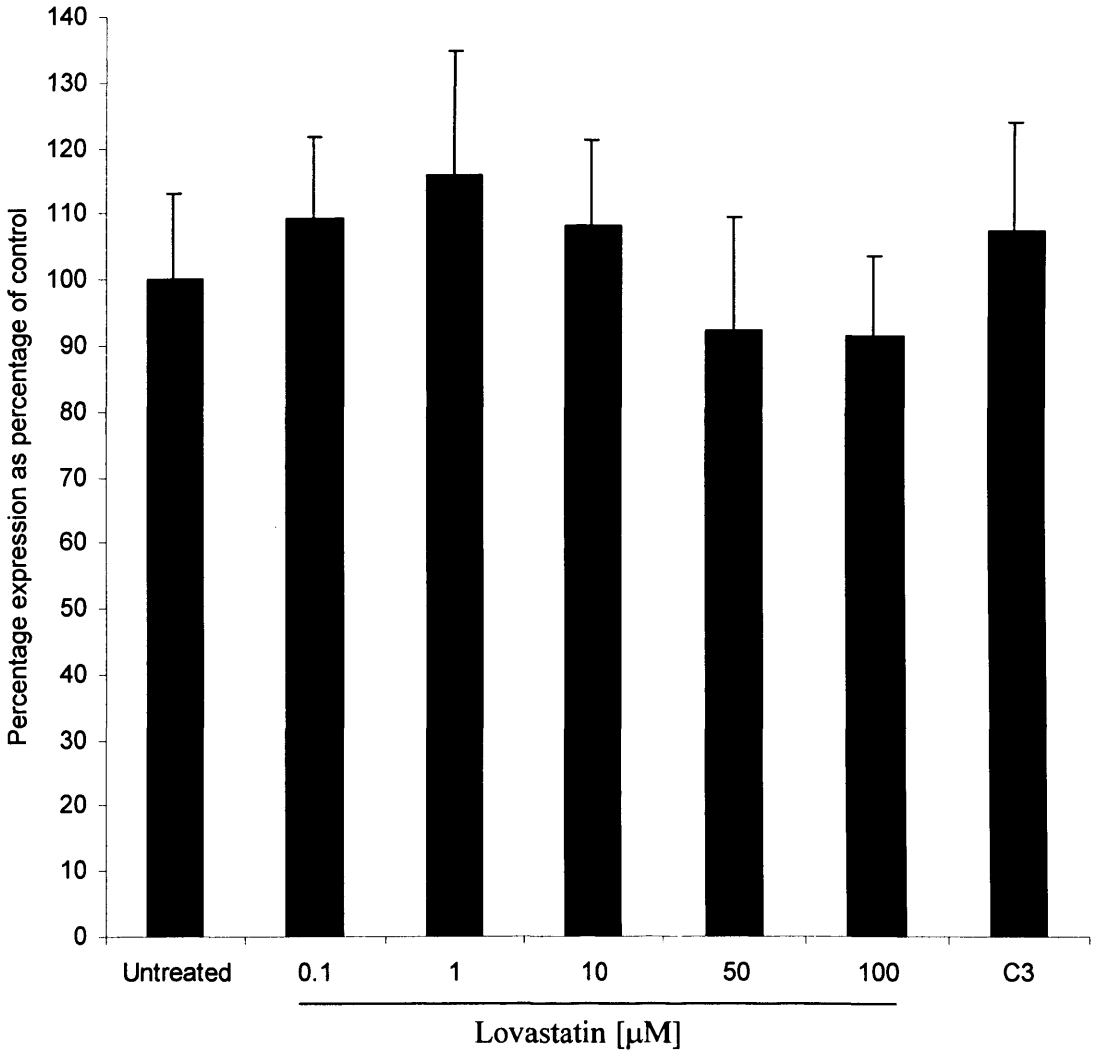

Figure 5.6 REC ICAM-1 expression is unaffected by lovastatin.
REC monolayers were pretreated with increasing concentrations of lovastatin [0-100μM] or C3 transferase [10μg/ml] for 24h and 16h respectively. Levels of ICAM-1 expression were determined using the 1A29 antibody by flow cytometry. Bars represent mean percentage of ICAM-1 positive cells as a percentage of control ICAM-1 expression. Bars represent means ± S.D. of three independent experiments.

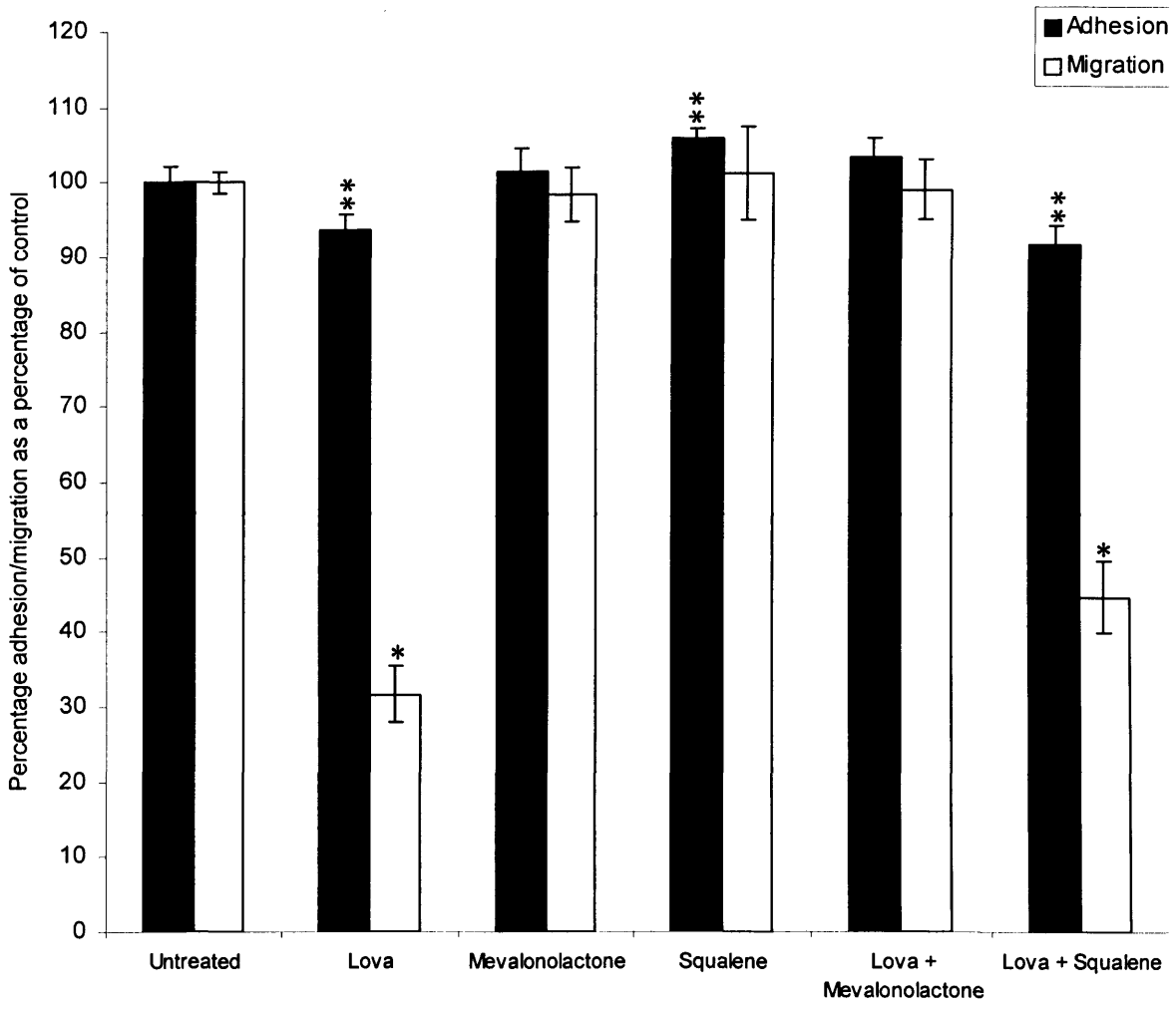

Figure 5.7 Concomitant administration of mevalonolactone but not squalene with lovastatin is able to reverse lovastatin-induced inhibition of lymphcoyte migration.

REC monolayers were pretreated for 24h with lovastatin, mevalonolactone or squalene alone or lovastatin in the presence of mevalonolactone or squalene. Means are expressed as a percentage of control adhesion or migration. Ten (migration) or twelve (adhesion) wells were assayed per condition per experiment. Each experiment was performed in triplicate. Results are expressed as the means ± SEM. Statistical analysis was performed using the Student's T-test. * $p< 0.001$, ** $p< 0.05$ as compared with controls.

to squalene treated monolayers was significantly increased to 105.7 ± 1.3% of control ($p \leq 0.05$). Levels of adhesion of PLN to REC monolayers treated with mevalonolactone alone and lovastatin in the presence of mevalonolactone did not differ from that of control. Pretreatment of REC monolayers with lovastatin resulted in a significant inhibition of antigen-specific migration as compared to control ($p \leq 0.001$) while antigen-specific T cell migration across mevalonolactone, squalene and lovastatin and mevalonolactone treated monolayers showed levels of migration comparable with that of control. However, monolayers pretreated with lovastatin and squalene exhibited a significant inhibition in lymphocyte TEM (44.6 ± 4.9%; $p<0.001$) as compared to controls.

Lovastatin inhibits lymphocyte migration across RPE monolayers

It is proposed that the BRB comprises of two barriers, the anterior BRB formed by the REC of the retinal vasculature and also the posterior BRB formed by the RPE of the choroid. It was therefore decided to investigate the effect of lovastatin upon lymphocyte TEM across an *in vitro* model using the previously characterised LD7.4 RPE cell line (Greenwood *et al* 1996). A mean level of migration across LD7.4 monolayers under control conditions was 31 ± 2.0% whilst mean adhesion to LD7.4 monolayers was 26.3 ± 2.3% (data not shown; Figure 5.8).

Lovastatin elicited a small but significant inhibition of PLN adhesion to RPE monolayers as compared with control (92.8 ± 3.0% vs 100 ± 2.0%; $p \leq 0.05$) while inhibition of antigen-specific T cell migration was comparable with that of the inhibition observed across lovastatin treated REC (23.9 ± 2.1% vs 100 ± 5.3%; $p \leq 0.001$).

Determination of active lovastatin

In our experiments inactive lovastatin was added to cultures and, due to the dose-dependent effects observed *in vitro*, it was important to quantitate the levels of active lovastatin hydroxy acid present within the cultures. The concentrations of lovastatin hydroxy acid were determined as described in chapter 2 (section 2.13.18).

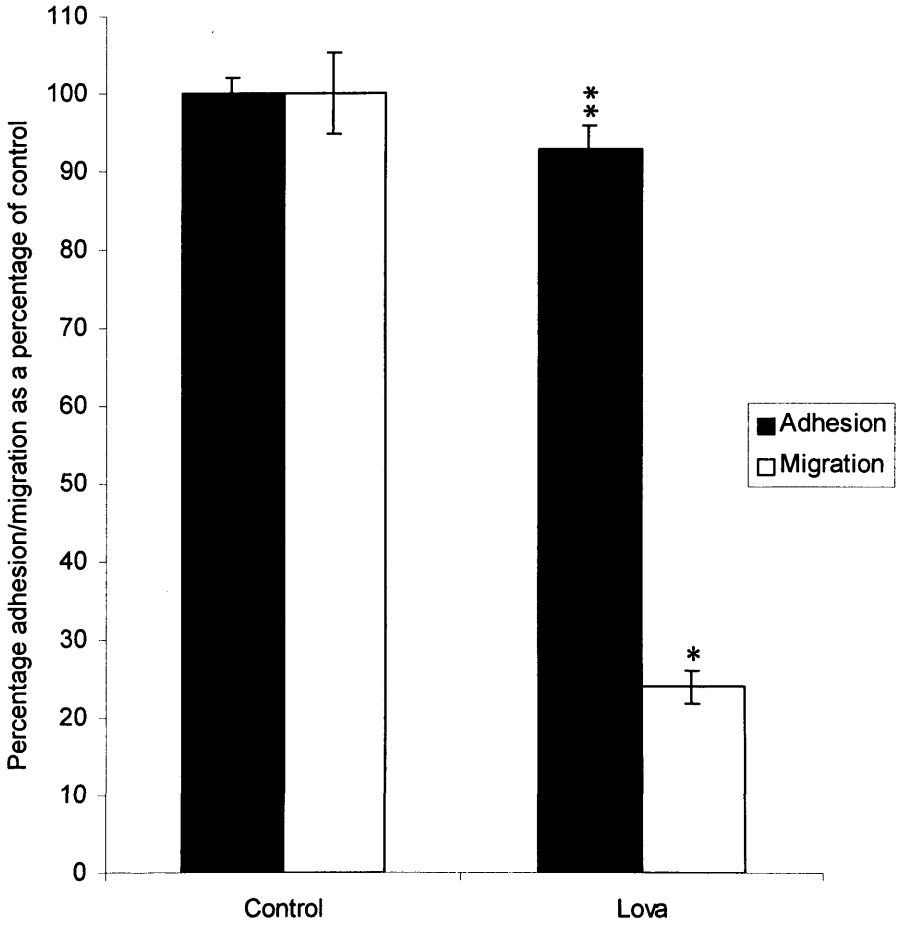

Figure 5.8 Pretreatment of RPE monolayers with lovastatin [10μM] for 24h results in significant inhibition of lymphocyte TEM and a small but significant effect upon lymphocyte adhesion.

RPE cells were pretreated with lovastatin [10μM] for 24h and PLN adhesion to and antigen specific TEM across the RPE cell line was assessed. Means are expressed as a percentage of control adhesion or migration. Ten (migration) or twelve (adhesion) wells were assayed per condition per experiment. Each experiment was performed in triplicate. Results are expressed as the means ± SEM. Statistical analysis was performed using the Student's T-test. *$p \leq 0.001$, **$p \leq 0.05$.

Results revealed that approximately 50% of total lovastatin added to each culture was present after 24h in the active lovastatin acid form (Table 5.2). This data would suggest that significant inhibition of lymphocyte TEM is achieved with 0.07μM lovastatin hydroxyacid.

Concentration of Lovastatin [μM]	Concentration of Lovastatin Acid [μM] ± SEM
0	0
0.1	0.07 ± 0.0
1	0.72 ± 0.01
10	6.43 ± 0.24
50	28.13 ± 2.01
100	51.30 ± 10.19

Table 5.2 approximately 50% of total lovastatin added to REC cultures is in the active lovastatin hydroxyacid form after 24h.
REC monolayers were pretreated with increasing concentrations of lovastatin [0-100μM] for 24h after which time supernatants were harvested and assayed for lovastatin hydroxy acid concentrations by means of LC/MS/MS (see chapter 2). Results are shown as the means of triplicate samples ± S.D.

Discussion

Given previous findings within the laboratory that statins were able to inhibit lymphocyte transmigration across the BBB we set about to determine whether similar mechanisms were employed at the BRB. Our data clearly demonstrates that lovastatin significantly inhibits lymphocyte TEM across both REC and RPE monolayers *in vitro*. This data also demonstrates that this inhibition of TEM is not as a result of the inhibition of the cholesterol synthesis pathway, but due to the specific inhibition of isoprenoid pyrophosphate intermediates and therefore the inhibition of active prenylated Rho and subsequent ICAM-1 signalling, which is known to be essential for migration across the BBB (Adamson *et al* 1999). It has previously been shown that another statin, pravastatin, inhibits neutrophil and monocyte migration across HUVECs *in vitro* in a mevalonate-dependent manner, which potentially supports a role for Rho dependent migration across HUVECs (Dunzendorfer *et al* 1997).

REC pre-treatment for 24h with increasing concentrations of lovastatin resulted in a highly significant inhibition of antigen-specific T cell TEM in a dose-dependent manner with similar effects previously described for lovastatin upon BEC (Greenwood *et al* 2003). From this data it was decided to investigate the effects of 10μM lovastatin as this showed significant inhibition of antigen-specific lymphocyte TEM without a significant loss of T cell or EC viability, thereby suggesting the inhibitory effects observed are as a result of the direct effects of lovastatin upon EC intracellular signalling pathways and not simply due to a loss in cell viability. Previous reports have suggested statin treatment to be both anti- (Tanaka *et al* 2004) and pro-apoptotic (Kaneta *et al* 2003, Erl *et al* 2003). In our present study we investigated cell viability as determined by PI exclusion and therefore did not investigate apoptosis *per se*. The concentration of lovastatin used in these studies has previously been used in the investigation of the effects of lovastatin upon TEM in an *in vitro* model of the BBB (Greenwood *et al* 2003).

Exposure of both EC and T cells to lovastatin during the period of a 90min adhesion assay or 4h migration assay showed no significant differences in the levels of PLN cell adhesion or antigen-specific T cell migration. This lack of inhibition of TEM at 4h suggests that a sufficient intracellular store of isoprenyl groups remains which must be utilized prior to the effects of lovastatin becoming evident (Adamson *et al* 1999). Given that migration and adhesion assays were carried out in the presence of lovastatin our data would suggest that lovastatin does not inhibit the ICAM-1-LFA-1 interaction as has previously been suggested. It has been suggested that statins may mediate their anti-inflammatory effect by binding directly to LFA-1 (Weitz-Schmidt *et al* 2001). The effects reported here for REC and RPE are consistent with the effects of lovastatin upon BEC monolayers (Greenwood *et al* 2003) and also for the duration required in order to achieve sufficient depletion of intracellular stores of isoprenoid groups (Adamson *et al* 1999).

A significant inhibition of antigen-specific T cell migration was found as a result of EC treatment with statins for 24h. We predict these effects are due to the inhibition of isoprenyl groups required for the generation of functional Rho and a subequent inhibtion of REC and RPE ICAM-1 signalling pathways. Here we show that cell surface expression of REC ICAM-1 is not affected by lovastatin, which is consistent with ICAM-1 expression by BEC in response to lovastatin treatment (Greenwood *et al* unpublished data) and that pre-treatment of both REC and RPE monolayers with lovastatin for 24h results in comparable levels of TEM inhibition. This data therefore supports a role for lovastatin in the inhibition of T cell migration through RPE monolayers and thus migration at the level of the posterior BRB. Our results also suggest that similar mechanisms may be employed in T cell migration across RPE and REC monolayers, both of which demonstrate a crucial role for ICAM-1 and intracellular ICAM-1 signalling.

Inhibition of TEM by C3 transferase, a known specific inhibitor of Rho proteins, was comparable with that of the inhibition achieved with pre-treatment of REC

monolayers with lovastatin [1μM]. This data supports a role for Rho proteins in the inhibition of T cell migration by lovastatin at the BRB.

Given our hypothesis, we set about to demonstrate that lovastatin specifically inhibits lymphocyte TEM as a result of the inhibition of isoprenoid pyrophosphates, intermediates of the cholesterol synthesis pathway, and not as a result of inhibition of cholesterol synthesis. Experiments were performed to determine the effects of exogenous mevalonolactone, a precursor of isoprenoid donating compounds and squalene, an intermediate of the cholesterol synthesis pathway downstream of the isoprenyl donating compounds at concentrations previously used with effects observed *in vitro* for mevalonolactone (Greenwood *et al* 2003) and squalene (Fisher *et al* 1999).

We found that inhibition of lymphocyte TEM by lovastatin was reversed by the addition of exogenous mevalonolactone but not as a result of the addition of exogenous squalene. This data supports our hypothesis that lovastatin-mediated inhibition of migration is not due to an inhibition of the cholesterol synthesis pathway, but is more specifically due to the inhibition of isoprenoid pyrophosphate donating compounds such as GPP and FPP, which are restored by the addition of exogenous mevalonolactone. This data is consistent with that previously reported for BEC (Greenwood *et al* 2003) and therefore suggests that similar mechanisms may be involved in lymphocyte migration across the BBB and BRB.

Pretreatment of EC for 24h with increasing concentrations of lovastatin shows small but statistically significant differences in the levels of PLN adhesion to REC monolayers. The biological significance of this reduction however, is undetermined. It is possible that the small reduction in adhesion is of functional relevance and that lovastatin treatment may elicit effects upon EC expression of cell adhesion molecules essential for PLN adhesion such as selectins and the Ig superfamily. However, as our studies demonstrate, lovastatin does not affect REC expression of ICAM-1. This may therefore suggest that lovastatin affects PLN adhesion to REC independently of

ICAM-1-mediated adhesion and, as such, may modulate the expression of molecules such as VCAM-1, known to play a role in lymphocyte adhesion to the vascular endothelium.

Previous findings within the laboratory showed that lovastatin is able to inhibit lymphocyte TEM across BEC *in vitro* and reduce lymphocyte traffic into the CNS in an experimental model of CNS inflammation, namely EAE (Greenwood *et al* 2003). Given that lovastatin is specifically able to inhibit lymphocyte TEM across REC and RPE *in vitro* and that similar mechanisms are involved in lymphocyte migration at the BRB and BBB, we therefore decided to investigate whether lovastatin is able to inhibit lymphocyte trafficking across the BRB *in vivo*. For this we used EAU as a model of human posterior uveitis.

Chapter 6

Immunomodulation of EAU by statins *in vivo.*

Introduction

As a result of the findings presented in chapter 5, that lovastatin treatment of REC *in vitro* could prevent or inhibit lymphocyte transmigration across the BRB, it was decided to test whether lovastatin could modulate lymphocyte trafficking *in vivo.*

Statins have previously been shown to modulate inflammatory cell infiltration into the CNS during EAE (Stanislaus *et al* 1999, Stanislaus *et al* 2001, Youssef *et al* 2002, Greenwood *et al* 2003, Aktas *et al* 2003). It was therefore decided to test whether statins were able to influence clinical progress of a $CD4^+$ T cell-mediated autoimmune inflammatory disease of the retina. It has previously been suggested that statins mediate the amelioration of clinical EAE by the induction of Th2 type or anti-inflammatory cytokines (Stanislaus *et al* 2001, Youssef *et al* 2002, Aktas *et al* 2003, Nath *et al* 2004). Studies from within our laboratory have provided evidence to suggest that statins may also alleviate disease through the inhibition of leukocyte migration (Greenwood *et al* 2003). Independent research also supports these findings, since it has been found that statins inhibit leukocyte migration into the CNS through the suppression of matrix metalloproteinase-9 (MMP-9; Neuhaus *et al* 2002, Wong *et al* 2001), known to be essential for the transmigration of leukocytes through the basal lamina (Goetzl *et al* 1996). Statin treatment has also been shown to inhibit the expression of both MHC class II antigen and co-stimulatory molecules on APC that are essential for stimulating autoimmune T-cells (Youssef *et al* 2002).

EAU is a well-characterised model for T cell-mediated retinal disease, which has been used for the characterisation of events involved in T cell infiltration across the BRB and into the retina. We therefore set out to test our hypothesis that lovastatin inhibits T cell trafficking across the BRB *in vivo* using the Lewis rat model of SAg peptide induced uveitis (Fling *et al* 1991). However, we were unable to induce reliable disease using this model and therefore decided to use a mouse model of EAU.

IRBP peptide$^{161-180}$ induced EAU in the B10.RIII mouse, previously characterised by Hankey *et al* (2001) is a reliable model of retinal disease which results in a high incidence and severity of disease (Silver *et al* 1995). Further characterisation of this model has revealed timepoints for the onset of lymphocyte infiltration and for the expected peak of disease thereby providing us with an extremely well characterised acute model of EAU, suitable for the assessment of immunomodulation of disease.

Aims

Having demonstrated that lovastatin is able to inhibt lymphocyte TEM *in vitro* (as presented in chapter 5) we set out to investigate whether lovastatin and another statin, atorvastatin, were able to modulate lymphocyte trafficking *in vivo*, using a mouse model of EAU.

Given the known pleiotropic effects of statins upon clinical disease including EAE we also investigated whether previously reported effects of statins including the induction of Th2 type cytokines and suppression of MHC class II expression were observed as a result of statin treatment of EAU.

Results

Effect of Lovastatin upon Lewis rat EAU

Initially we investigated the effects of lovastatin using a rat *in vitro* model of the BRB we therefore set out to investigate the effects of lovastatin upon Lewis rat SAg peptide induced EAU. SAg peptide induced EAU [75μg] was induced following the method of Fling *et al* (1991), and lovastatin therapy was initiated prior to the expected onset of lymphocyte infiltration at day 6 post immunisation. Eyes were assessed for clinical signs of disease daily by two independent observers from the onset of lovastatin therapy until the predicted peak of disease, day 15 post immunisation. However no detectable clinical disease was observed in the eyes of vehicle treated animals. To confirm our findings, we continued by investigating vascular disease of these animals by FA (day 12; Figure 6.1 A-C, see chapter 2 section 2.13.3) and also retinal disease (see chapter 2, section 2.13.7) by toluene blue staining of semi-thin sections of retina post enucleation at peak disease (Figure 6.1 D-F). Using these two parameters no clinical disease was determined in vehicle treated animals (Figure 6.1 B and E). Subsequent experiments set about to determine the effects of increasing amounts of peptide [0-600μg] upon the induction of EAU however no clinical, vascular or retinal disease was detectable at each of the doses investigated. As we were unable to induce disease by means of active immunisation with peptide we attempted to adoptively transfer EAU using uveitogenic T cells from actively immunised animals.

4-6wk old female Lewis rats were actively immunised with SAg peptide [75μg] and 10 days post immunisation PLN T cells were isolated and cultured in the presence of SAg peptide following the method of Sedgewick *et al* (1989; see chapter 2 section 2.6.5) in order to establish uveitogenic SAg peptide T cell lines. 48h prior to adoptive transfer of antigen-specific T cell line cells, cells were restimulated with antigen and feeders (see chapter 2, section 2.13.9). Naïve 4-6wk old female Lewis rats were then immunised with either $1x10^6$ or $5x10^6$ activated SAg peptide specific T

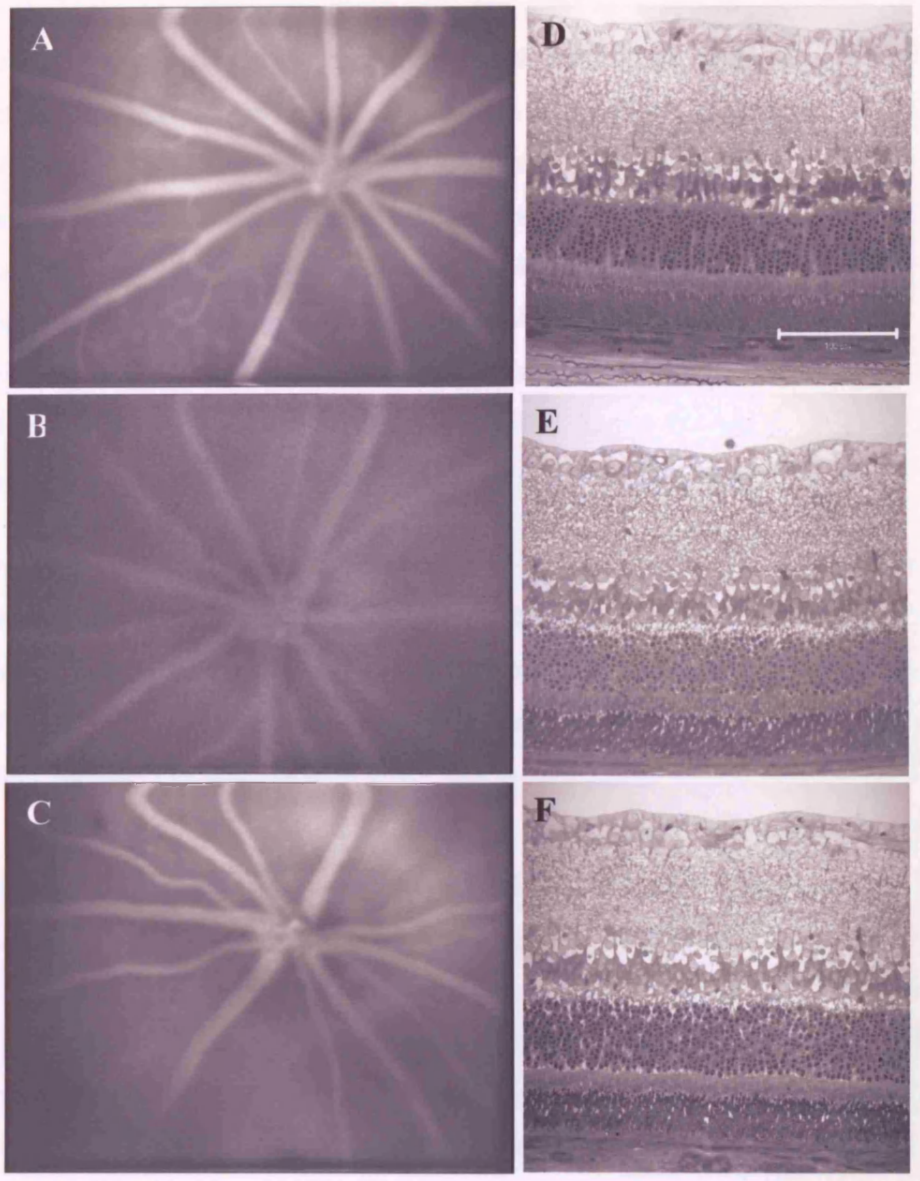

Figure 6.1 SAg peptide induced EAU in the Lewis Rat.
Panels A-C show vascular caliber of a normal control, vehicle treated and lovastatin treated retina respectively 12 days post immunization as determined by fluorescein angiography using a scanning laser ophthalmoscope. Panels D-F are representative light micrographs of toluidine blue stained histological semi-thin sections taken using a LSM 510 confocal microscope operating in transmission mode using a FITC configuration. A normal control (D), vehicle treated (E) and lovastatin treated (F) retina are shown. No signs of vascular leakage were detected as a result of fluorescein angiography, and no sign of disease was assessed daily from transfer of T cells to expected peak disease between day 13 and 15. Clinical grading of retinal disease was evident as determined by classical histology. Bar represents 100μM.

cells. Animals were then monitored daily for signs of adoptively transferred ocular inflammation. Clinical disease was assessed daily from transfer of T cells to expected peak disease between day 13 and 15. Clinical grading of disease was performed by two independent observers, however no clinical signs of disease were evident. To confirm these findings, vascular disease was assessed by FA and retinal disease determined by histology as described previously. No evidence of inflammation was detectable using either FA or classical histology. As these experiments were carried out following established protocols but no clinical disease was detected, we suspect that modifications to the peptide may have occurred during preparation, which may have altered the uveitogenic nature of the peptide.

Given our desire to test our hypothesis *in vivo,* that statins are able to modulate lymphocyte trafficking through the specific inhibition of ICAM-1 signalling, we decided to investigate the effects of lovastatin and atorvastatin in the B10.RIII mouse model of EAU in order to determine whether variations exist in the efficacy of different statins. For this purpose EAU was induced in 5-7 week old B10.RIII male mice by subcutaneous injection of 25μg of human IRBP peptide in incomplete freunds adjuvant supplemented with 60μg/ml Mycobacterium Tuberculosis (Hankey *et al* 2001). Daily treatment regimens (see chapter 2 section 2.13.11) were applied to each clinical group 5 days post immunisation until the expected peak of disease at day 12. Lovastatin treatment was given by intraperitoneal injection while atorvastatin was administered by oral gavage. Intraperitoneal injection of DMSO:PBS vehicle served as a control for lovastatin-treated animals while oral administration of PBS served as a control for atorvastatin-treated animals. Clinical disease was assessed daily from day 5 onwards with FA used to determine vascular disease at day 10 post immunisation. Retinal disease, as quantitated by classical histology, was graded at expected peak of disease (day 12). All subsequent experiments were carried out using this mouse model.

Lovastatin but not atorvastatin treatment of EAU ameliorates signs of ocular clinical disease

Clinical scoring of each treatment group was assessed daily until the predicted onset of peak disease. Mean clinical score of each treatment group was calculated at peak disease as the mean of both eyes. Normal animals presented with bright eyes with a good red reflex. Vehicle-treated animals in contrast showed signs of inflammation from day 9 which increased in severity to day 12. Signs of inflammation included clouding of the eye and a poor red reflex (Figure 6.2 A and C). Lovastatin-treated EAU induced animals presented with minimal signs of inflammation and generally exhibited a good red reflex (Figure 6.2 B and D). Lovastatin and mevalonolactone-treated animals showed signs of inflammation comparable with that of vehicle-treated controls whilst lovastatin and squalene-treated animals showed minimal signs of inflammation, which was comparable with that of lovastatin-treated animals.

Clinical grading of animals by two independent observers revealed that lovastatin treatment at both concentrations resulted in a significant reduction in disease incidence ($p \leq 0.001$), mean group score ($p \leq 0.001$) and mean EAU score ($p \leq 0.01$) as compared to vehicle-treated animals (Table 6.1). Lovastatin and mevalonolactone treatment of EAU animals showed a small but significant decrease in mean group score ($p \leq 0.05$) and also mean EAU score ($p \leq 0.01$) as compared with vehicle-treated animals, however incidence of disease ($p \leq 0.01$) and mean group scores ($p \leq 0.01$) were found to be significantly greater than that of lovastatin-treated animals (Table 6.1). Lovastatin and squalene-treated animals showed a significant reduction in disease incidence ($p \leq 0.001$) and mean group score ($p \leq 0.001$) as compared to vehicle-treated animals, whilst no significance was detectable as compared to lovastatin-treated animals (Table 6.1).

In contrast atorvastatin treated animals showed no significant difference in disease incidence or disease severity as compared to vehicle gavage controls (Table 6.1).

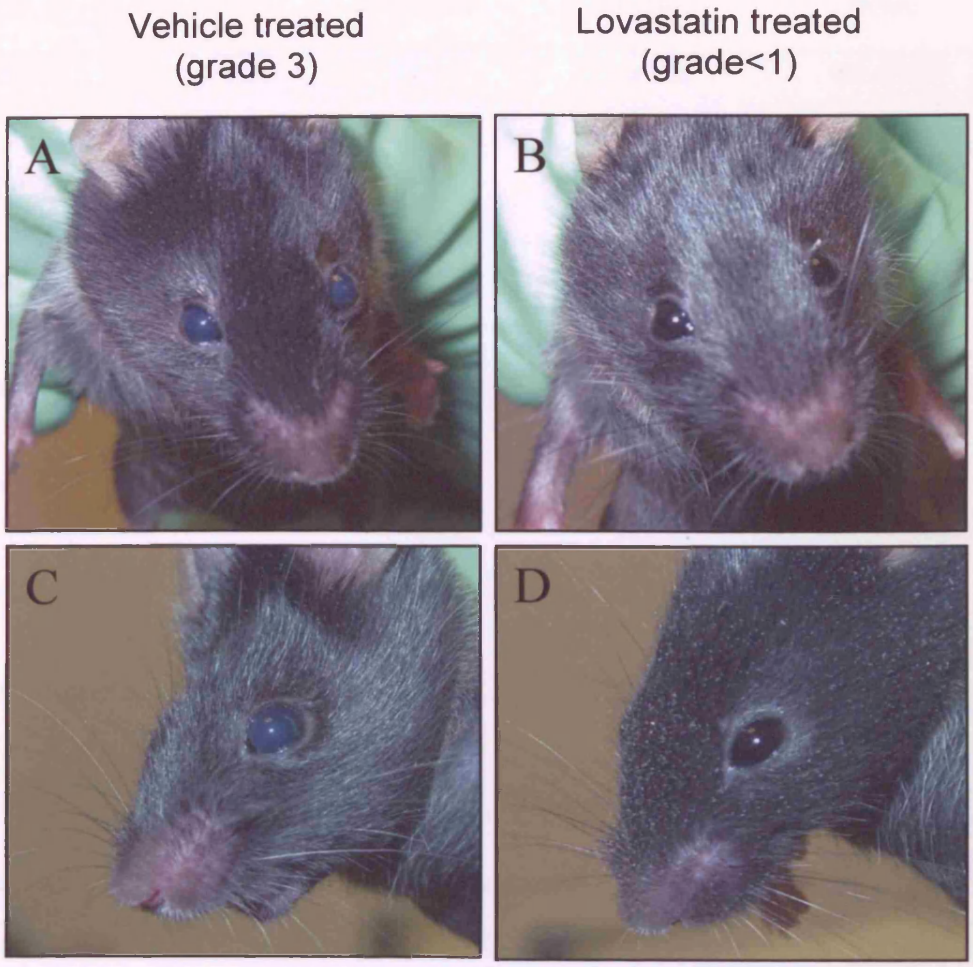

Figure 6.2 Clinical EAU.
Clinical symptoms of disease were graded following criteria previously described (Chapter 2). Panels A and C show a vehicle treated animal with grade 3 disease. Panels B and D show an animal treated with lovastatin [20mg/kg/day].

Treatment	No. EAU/total	Mean Group Score	Mean EAU Score
Normal	0/12	0.0 ± 0.0	0.0 ± 0.0
Vehicle treated (i.p)	23/26	2.88 ± 0.31	3.26 ± 0.25
Lovastatin [20 mg/kg/day]	8/27***	0.59 ± 0.20***	2.00 ± 0.33**
Lovastatin [2 x 20 mg/kg/day]	0/5***	0***	0
Lovastatin + mevalonolactone	11/13ξξ	1.54 ± 0.35*ξξ	1.82 ± 0.35**
Lovastatin + squalene	0/6***	0 ***	0
Vehicle treated (gavage)	7/8	3.38 ± 0.56	3.86 ± 0.34
Atorvastatin [10 mg/kg/day]	13/18	2.22 ± 0.40	3.08 ± 0.31

Table 6.1 Mean clinical score of EAU day 12 post immunisation.
Clinical disease was graded following criteria described in chapter 2. Incidence of EAU is calculated as the number of animals, which display clinical signs of disease as a proportion of the total number of animals within the experimental group. The mean group score is calculated as the mean disease score of all animals within the experimental group, while the mean EAU score is determined as the mean of all animals, which showed signs of clinical disease. Data from vehicle and lovastatin-treated mice are means from four separate experiments. Data from lovastatin and mevalonolactone-treated and atorvastatin-treated mice are from two separate experiments. Data from lovastatin [2 x 20 mg/kg/day], lovastatin and squalene are from one experiment. Statistical analysis was performed using the Mann-Whitney test. * $p \leq 0.05$ compared to vehicle control, ** $p \leq 0.01$ compared to vehicle control, *** $p \leq 0.001$ compared to vehicle control and ξξ $p \leq 0.01$ compared to lovastatin.

Lovastatin but not atorvastatin treatment of EAU ameliorates signs of retinal vascular disease

Vascular disease was assessed by means of FA and a fundus camera (see chapter 2 section 2.13.12). Photographs of the retina were captured 3 min (early angiograms) and 5-8 min (late angiograms) post intraperitoneal injection of sodium fluorescein [2%]. Fluorescein angiograms were scored blind by Hadi Zambarakji. Control animals showed no signs of vascular leakage (Figure 6.3A). Early and late angiograms of vehicle-treated animals show significant levels of hyperfluorescence at the optic disc and dilation of blood vessels (Figure 6.3 B and C). Lovastatin-treated animals showed no signs of vascular dilation or hyperfluorescence in both early and late angiograms (Figure 6.3 D and E). Lovastatin and mevalonolactone-treated animals showed signs of vascular dilation and hyperfluoresence (Figure 6.3 F). Minimal signs of vascular dilation and hyperfluorescence were observed in the lovastatin and squalene-treated group. Fluorescein angiograms of atorvastatin and vehicle (gavage) treated animals revealed marked vascular dilation and hyperfluorescence (data not shown).

Clinical grading of the fluorescein angiograms revealed that lovastatin treatment of EAU resulted in a significant reduction in disease incidence ($p \leq 0.01$) and also mean group score ($p \leq 0.001$) as compared to vehicle-treated animals (Table 6.2). Lovastatin and mevalonolactone-treated animals presented with clinical disease comparable with that of vehicle-treated animals, however disease incidence ($p \leq 0.01$) and mean group score ($p \leq 0.01$) were found to be significantly greater than that of lovastatin-treated animals (Table 6.2). Lovastatin and squalene-treated animals showed a significant difference in mean group score ($p \leq 0.05$) as compared with vehicle control, however the reduction in disease incidence in this group did not achieve significance. No significant differences were detectable as compared with lovastatin-treated animals (Table 6.2).

Atorvastatin-treated animals showed no significant differences as compared to vehicle-treated animals by each of the three parameters assessed (Table 6.2).

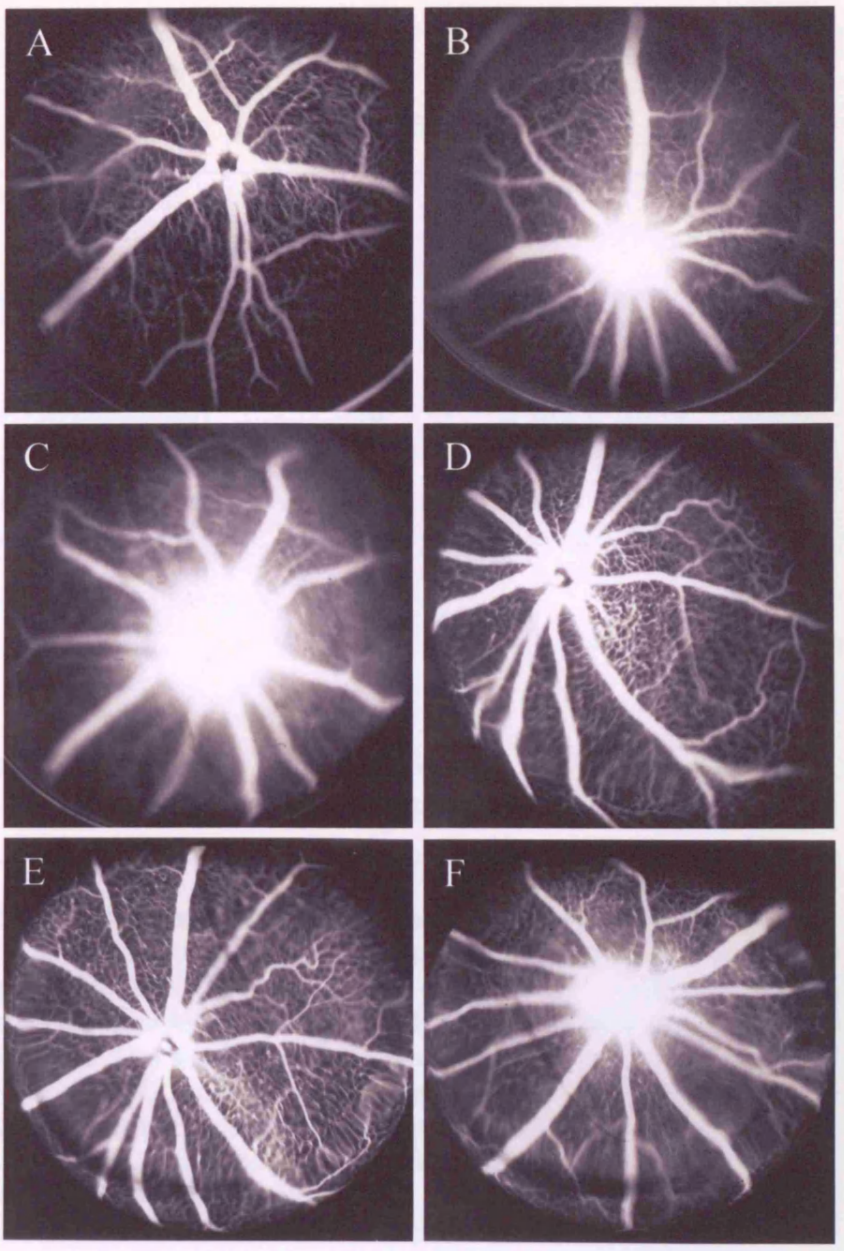

Figure 6.3 Representative images of retinal vessels during FA of normal, vehicle and lovastatin treated animals.
Clinical disease as determined by vascular leakage was graded following criteria previously described (Chapter 2). Panel A shows an early angiogram from a normal control animal. Panel B and C show an early and late angiogram of a vehicle (i.p.) treated animal respectively. This animal was determined to have grade 3 disease. Panels D and E show early and late angiograms from a lovastatin treated animal. This animal was given grade 0. Panel F shows an early angiogram from a lovastatin and mevalonolactone treated animal. This animal was scored with grade 3 disease.

Treatment	No. EAU/total	Mean Group Score	Mean EAU score
Normal animals	0/3	0	0
Vehicle treated	9/13	1.38 ± 0.33	2.00 ± 0.33
Lovastatin [20 mg/kg/day]	1/10**	0.10 ± 0.10***	1.00 ± 0.00
Lovastatin + mevalonolactone	5/6ξξ	1.33 ± 0.42ξξ	1.60 ± 0.40
Lovastatin + squalene	2/6	0.33 ± 0.21*	1.00 ± 0.00
Atorvastatin [10 mg/kg/day]	4/6	0.67 ± 0.21	1.00 ± 0.00

Table 6.2 Mean vascular leakage scores as determine by FA.
FA was performed at day 10 post immunisation. Vascular leakage was graded following criteria described previously (Chapter 2). Incidence of leakage is calculated as the number of animals which display vascular leakage of disease as a proportion of the total number of animals within the experimental group assessed by FA. The mean group score is calculated as the mean score of the animals assessed within the experimental group, while the mean EAU score is determined as the mean of all animals which showed signs of vascular leakage. Data from vehicle and lovastatin-treated mice are means from four separate experiments. Data from lovastatin and mevalonolactone-treated and atorvastatin-treated mice are from two separate experiments. Data from lovastatin [2 x 20 mg/kg/day], lovastatin and squalene are from one experiment. Statistical analysis was performed using the Mann-Whitney test. *$p \leq 0.05$ compared to vehicle control, *** $p \leq 0.001$ compared to vehicle control, ξξ $p \leq 0.01$ compared to lovastatin.

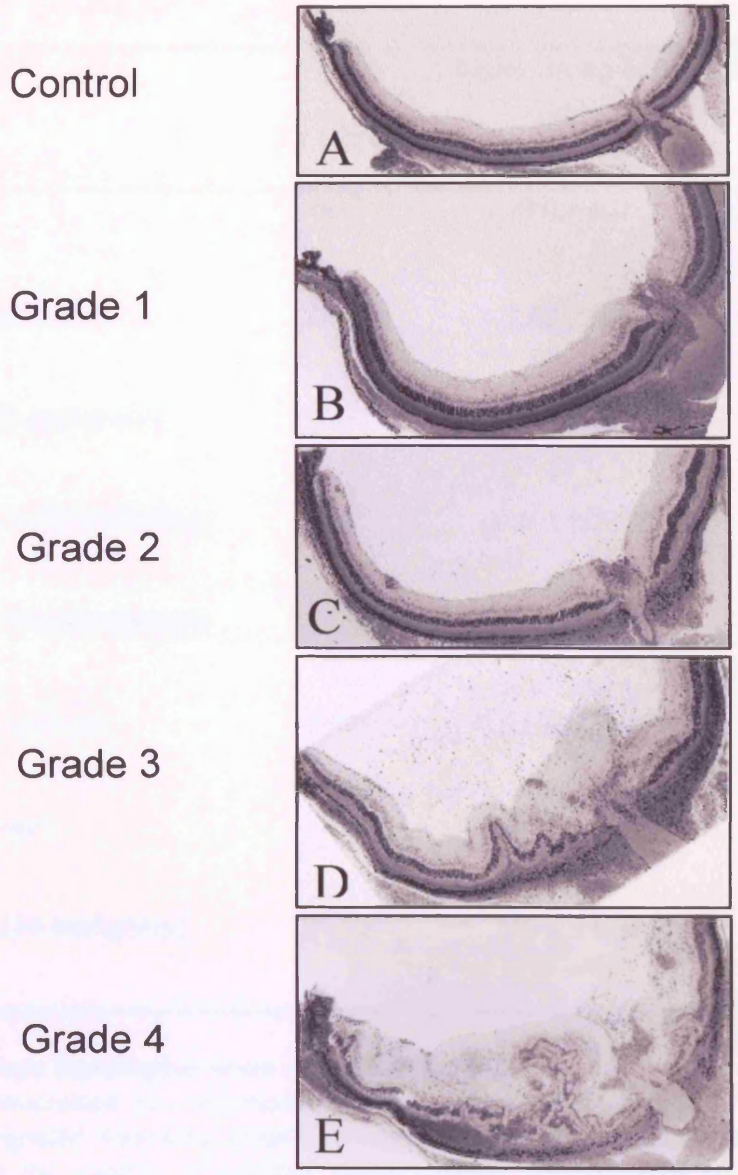

Figure 6.4 Representative images of EAU histology.
Eyes were enucleated at peak disease and histological disease was graded following criteria described in chapter 2. Panel A shows a normal retina grade 0, panel B shows a retina isolated from a lovastatin treated animal, grade 1. Panel C shows a retina isolated from a lovastatin and mevalonolactone treated animal, while panels D and E show retinae from vehicle treated animals of grade 3 and 4 respectively.

Treatment	No. EAU/total	Mean Group Score	Mean EAU Score
Normal	0/12	0.0 ± 0.0	0.0 ± 0.0
Vehicle treated (i.p)	25/26	3.42 ± 0.31	3.56 ± 0.29
Lovastatin [20 mg/kg/day]	15/27**	1.44 ± 0.31***	2.60 ± 0.32*
Lovastatin [2 x 20 mg/kg/day]	2/5*	0.40 ± 0.24***	1.00 ± 0.00
Lovastatin + mevalonolactone	13/13ξξ	2.54 ± 0.39*ξ	2.54 ± 0.39*
Lovastatin + squalene	1/6**	0.33 ± 0.33 ***	2.00 ± 0.00
Vehicle (gavage)	8/8	4.25 ± 0.49	4.25 ± 0.49
Atorvastatin [10 mg/kg/day]	18/18	3.44 ± 0.32	3.44 ± 0.32

Table 6.3 Mean histological score of retinal disease.
Eyes were enucleated and immersion fixed in Karnovsky's fixative. Histological disease was graded following criteria described (Chapter 2). Incidence of EAU is calculated as the number of animals which display clinical signs of disease as a proportion of the total number of animals within the experimental group. The mean group score is calculated as the mean disease score of all animals within the experimental group, while the mean EAU score is determined as the mean of all animals which showed signs of clinical disease. Data from vehicle and lovastatin-treated mice are means from four separate experiments. Data from lovastatin and mevalonolactone-treated and atorvastatin-treated mice are from two separate experiments. Data from lovastatin [2 x 20 mg/kg/day] and lovastatin and squalene is from one experiment. Statistical analysis was performed using the Mann-Whitney test. * $p \leq 0.05$ compared to vehicle control, ** $p \leq 0.01$ compared to vehicle control, *** $p \leq 0.001$ compared to vehicle control, ξ $p \leq 0.05$ compared to Lovastatin, ξξ $p \leq 0.01$ compared to Lovastatin.

Statin treatment does not affect serum cholesterol concentration

We next investigated the effects of our dosing regimen upon serum cholesterol concentration in the different experimental groups. Lovastatin treatment of EAU did not significantly affect serum cholesterol levels as compared to vehicle-treated animals or normal controls (Figure 6.5). However lovastatin and squalene-treated animals were found to exhibit a small but significant increase in serum cholesterol as compared with vehicle-treated controls ($p \leq 0.05$), while atorvastatin-treated animals showed a significant increase in serum cholesterol as compared with normal controls ($p \leq 0.05$; Figure 6.5).

Lovastatin but not atorvastatin treatment reduced total splenocyte number

Data presented here clearly supports a role for statin therapy in the amelioration of EAU. Given our *in vitro* findings outlined in chapter 5 and the results presented here, we hypothesise that amelioration of disease is due to the inhibition of ICAM-1 signalling, and therefore an inhibition of lymphocyte trafficking into the CNS. This theory is strongly supported by the rescue of inhibition of T cell migration both *in vivo* and *in vitro* (chapter 5) by the addition of exogenous mevalonolactone but not squalene. Previous reports have suggested that statins may influence T cells directly (Youssef *et al* 2002, Stanislaus *et al* 2002, Neuhaus *et al* 2002, Hillyard *et al* 2002, Aktas *et al* 2003, Leung *et al* 2003, Nath *et al* 2004) by the modulation of T cell effector mechanisms. We therefore investigated the effects of statins upon T cells in our system.

Our first observation was that lovastatin treatment of EAU induced animals resulted in a significant reduction in total splenocyte number ($p \leq 0.01$) as compared with vehicle-treated controls (Figure 6.6 A). Both once and twice daily treatment with lovastatin resulted in a highly significant reduction in splenocyte number as compared with vehicle-treated animals ($p \leq 0.001$ and $p \leq 0.01$ respectively; Figure 6.6 A). Lovastatin and mevalonolactone treatment resulted in a significant decrease in cell numbers as compared with vehicle-treated animals ($p \leq 0.05$), while lovastatin and squalene therapy resulted in a significant increase in cell numbers ($p \leq 0.05$) as compared with lovastatin treatment alone.

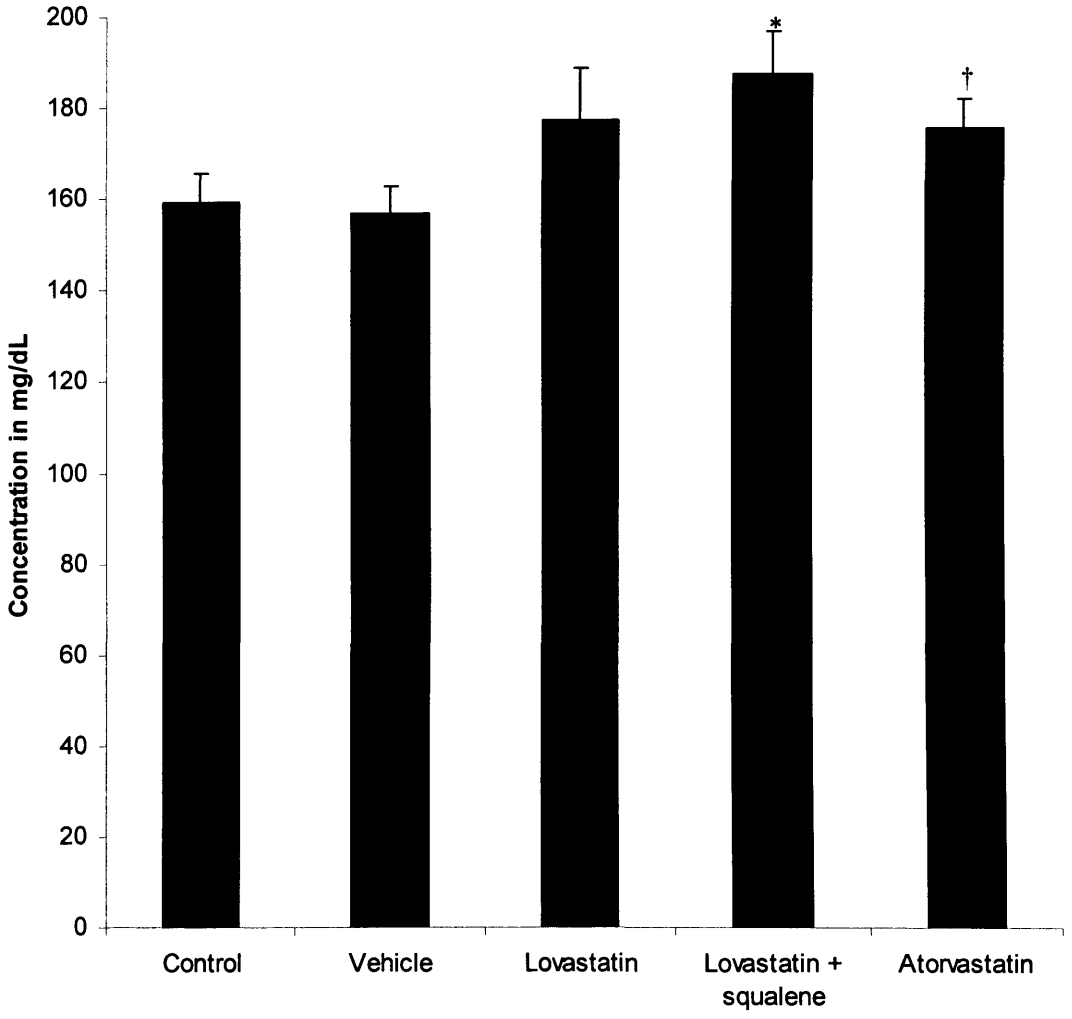

Figure 6.5 Histogram of mean serum cholesterol levels in control, vehicle, lovastatin, lovastatin and squalene and atorvastatin treated individuals.
Cholesterol levels were determined using the Infinity™ Cholesterol liquid stable reagent kit (as described in chapter 2). Statistical analysis was performed using the Mann-Whitney test and statistical significance was detected as follows; * $p \leq 0.05$ as compared with vehicle controls and † $p \leq 0.05$ compared with normal controls.

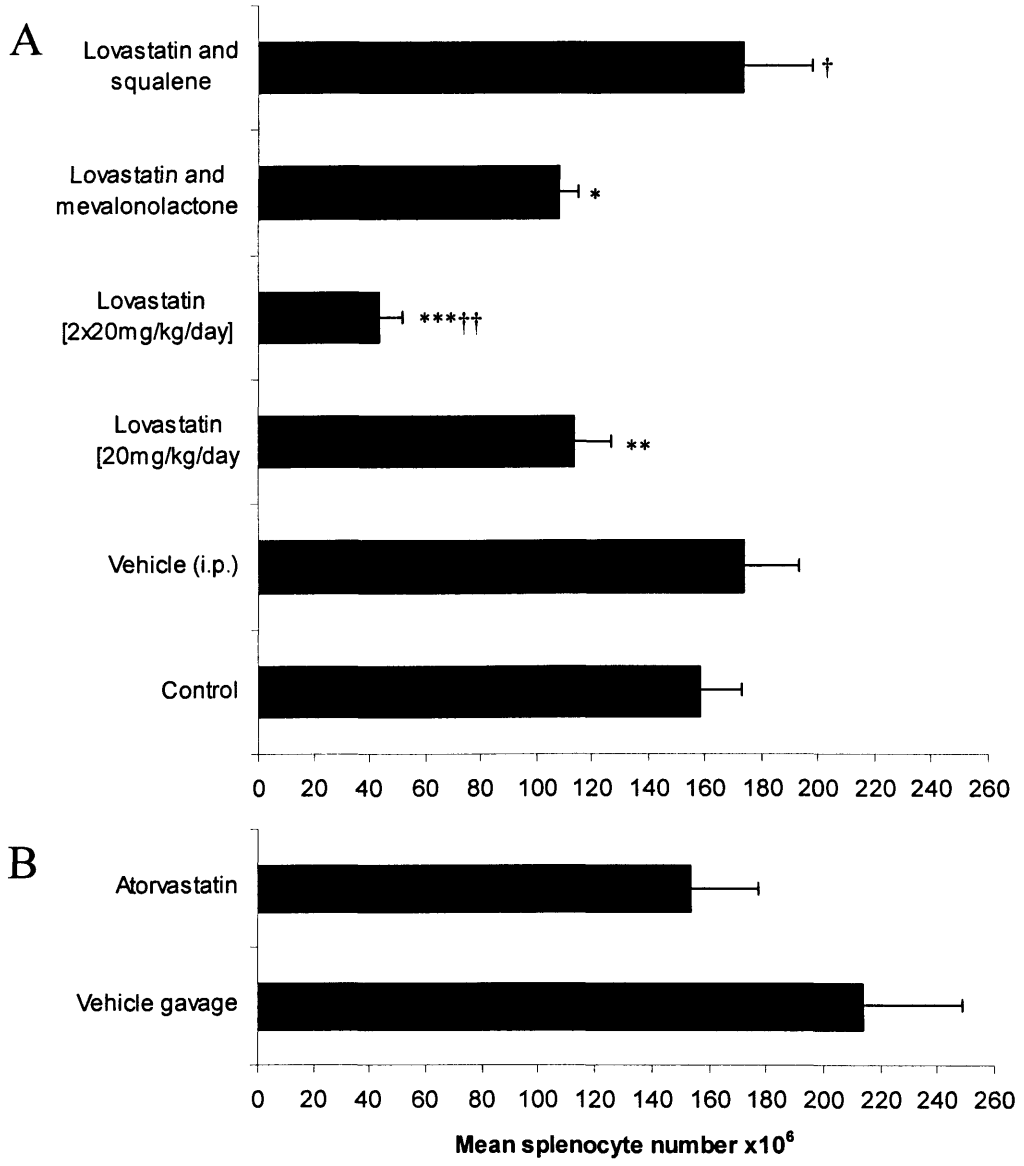

Figure 6.6 Lovastatin treatment results in a significant reduction of total splenocyte number but atorvastatin therapy does not.
Panel A shows splenocyte number from lovastatin and lovastatin-related-treated animals. Panel B shows splenocyte number of atorvastatin-treated animals. Total viable splenocyte number was determined using a haemocytometer and trypan blue exclusion. Statistical analysis was performed using the Mann-Whitney U test. * $p \leq 0.05$ ** $p \leq 0.01$ and ***$p \leq 0.001$ as compared to vehicle control, † $p \leq 0.05$, †† $p \leq 0.01$ as compared to lovastatin (20mg/kg/day).

Atorvastatin therapy in contrast showed no significant effect upon total splenocyte number as compared with vehicle gavage-treated controls (Figure 6.6B).

Lovastatin but not atorvastatin treatment results in an inhibition of T cell proliferation

Splenocyte proliferation was assessed immediately *ex vivo* by means of a proliferation assay (see chapter 2, section 2.13.13). Splenocytes isolated from normal control animals showed no significant proliferation in response to antigen, although the response to Con A was positive (data not shown). Incubation of splenocytes isolated from vehicle, lovastatin, lovastatin and mevalonolactone, lovastatin and squalene and atorvastatin-treated animals with antigen in culture resulted in a dose-dependent increase in proliferation as compared with unstimulated cultures of the same experimental group ($p \leq 0.05$, $p \leq 0.01$; Figure 6.7 A). Constitutive (unstimulated) splenocyte proliferation of the lovastatin, lovastatin and mevalonolactone and lovastatin and squalene groups was significantly lower than that of vehicle (i.p.) controls ($p \leq 0.05$ and $p \leq 0.01$ respectively) while constitutive proliferation of splenocytes from the atorvastatin-treated group showed no difference as compared with that of the vehicle gavage group (Figure 6.7 B). Splenocyte responses to antigen were reduced as a result of lovastatin and mevalonolactone ($p \leq 0.01$), lovastatin and squalene treatment ($p \leq 0.01$) and lovastatin alone although lovastatin treatment did not achieve significance. Lovastatin and mevalonolactone-treated splenocyte proliferation but not lovastatin and squalene-treated splenocytes proliferation in response to antigen was significantly lower than that observed in lovastatin-treated cultures alone ($p \leq 0.05$). This data suggests that lovastatin therapy reduces the proliferative capacity of splenocytes in response to antigen and ConA (data not shown). Cultures were also incubated with an anti-MHC class II antibody to determine whether antigen presentation within these cultures was MHC class II restricted (Figure 6.7 A and B). Culture of splenocytes in the presence of an anti-MHC class II antibody resulted in a significant inhibition of splenocyte proliferation in vehicle ($p \leq 0.01$), lovastatin ($p \leq 0.01$) and atorvastatin ($p \leq 0.01$) treated splenocytes. Anti-MHC class II antibody-mediated suppression of proliferation in response to antigen failed to reach significance in normal control cultures (data not shown). This

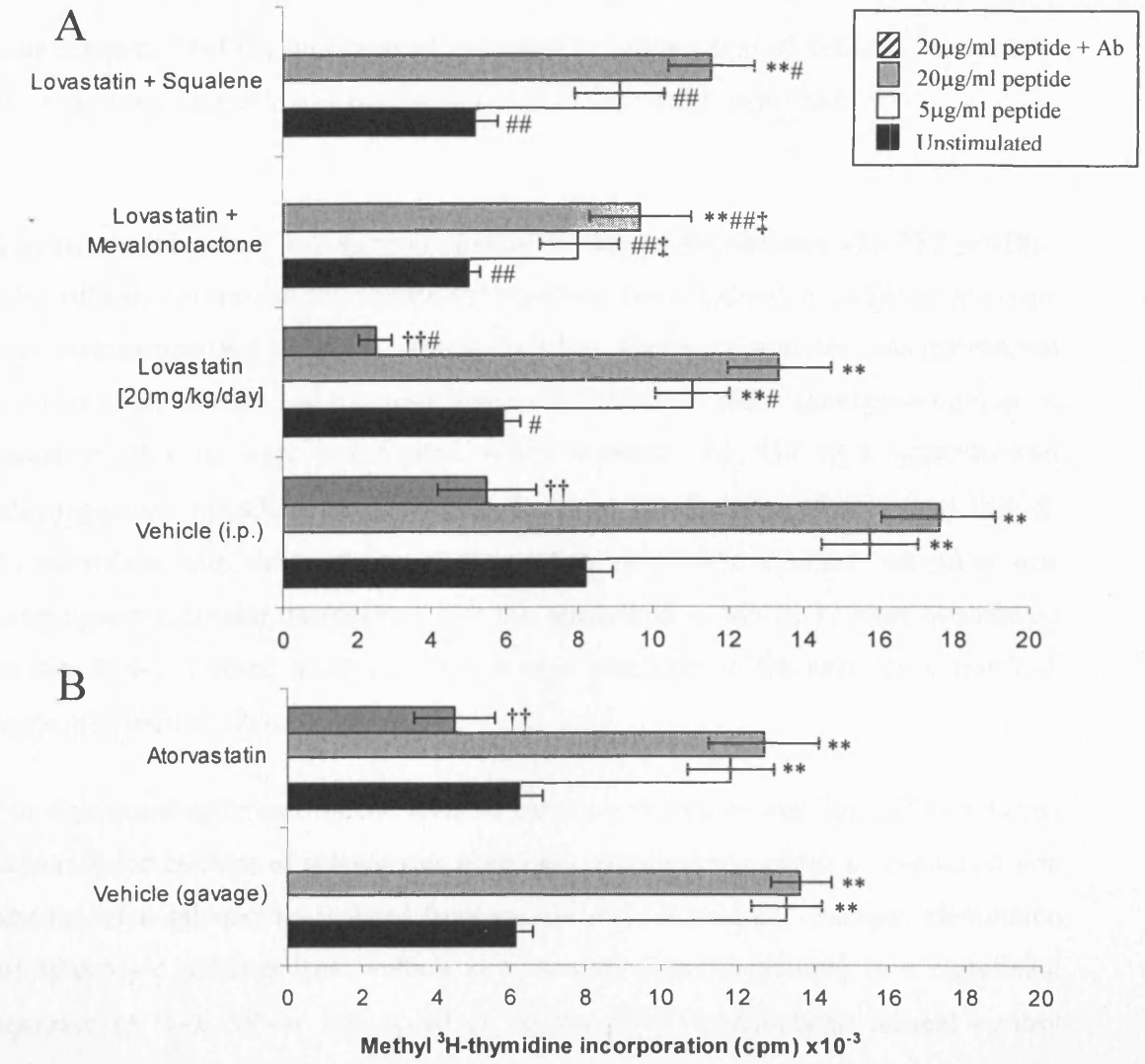

Figure 6.7 Lovastatin but not atorvastatin treatment results in a significant inhibition of splenocyte proliferation.

Panel A shows splenocyte proliferation of lovastatin and lovastatin related cultures while panel B shows splenocyte proliferation of atorvastatin cultures. Splenocyte proliferation is increased in primed splenocyte populations in response to antigen stimulation. Proliferation of primed splenocyte populations is inhibited by the incorporation of an anti-MHC class II blocking antibody. Splenocytes were cultured at $5x10^6$ cells/ml in the presence or absence of IRBP peptide for 72h. Proliferation of splenocytes was quantitated by methyl-^{3}H thymidine incorporation and scintillation counting. Statistical analysis was performed using the Mann-Whitney U test. Statistical analysis revealed, * $p \leq 0.05$, ** $p \leq 0.01$, as compared to unstimulated control within group, † $p \leq 0.05$, †† $p \leq 0.01$ 20µg/ml peptide plus antibody as compared with 20µg/ml peptide alone, ‡ $p \leq 0.05$, as compared with lovastatin alone and # $p \leq 0.05$, ## $p \leq 0.01$ as compared with vehicle alone. Splenocyte proliferation in the presence of MHC class II antibody was not performed for vehicle gavage, lovastatin and mevalonolactone and lovastatin and squalene-treated cultures.

data suggests that the proliferation observed in antigen primed cultures i.e. vehicle, lovastatin and atorvastatin-treated cultures is MHC class II restricted.

Lovastatin treatment ameliorates clinical disease in the absence of a Th2 profile

The influence of the different treatment regimens upon splenocyte cytokine secretion was investigated (see chapter 2, section 2.13.14). Cytokine secretion was quantitated for individual animals and the mean levels calculated for each experimental group. a panel of cytokines were investigated, which included Th1, Th2 type cytokines and also regulatory cytokines: IL-2, IL-4, IL-5, IL-10, IL-12, IFN-γ, TNF-α and TGF-β. In accordane with the methods of Youssef *et al* (2002), cytokine secretion was investigated at similar timepoints. For this purpose IL-2 and IL-12 were quantitated at 48h, IFN-γ, TNF-α, IL-10 and TGF-β were measured at 72h while IL-4 and IL-5 were measured at 120h.

No significant difference in the level of cytokine secretion was detectable between unstimulated cultures of splenocytes from each experimental group as compared with unstimulated splenocytes isolated from normal control animals. Antigen stimulation of splenocyte cultures from vehicle (i.p.)-treated animals resulted in a significant increase in IL-2, IFN-γ and IL-10 as compared to unstimulated normal control cultures ($p \leq 0.01$) however no increase in TNF-α secretion was detectable (Figure 6.8). In contrast, antigen-specific stimulation of splenocytes isolated from lovastatin treated animals resulted in a significant increase in IL-2 ($p \leq 0.01$) and IFN-γ ($p \leq 0.001$) secretion as compared to their respective unstimulated controls. No significant differences were detectable in the levels of TNF-α in response to antigen stimulation as compared to unstimulated controls. Similarly IL-10 production in response to antigen stimulation of lovastatin treated splenocytes was not detected (Figure 6.8). Lovastatin treatment however, resulted in a significant decrease in IFN

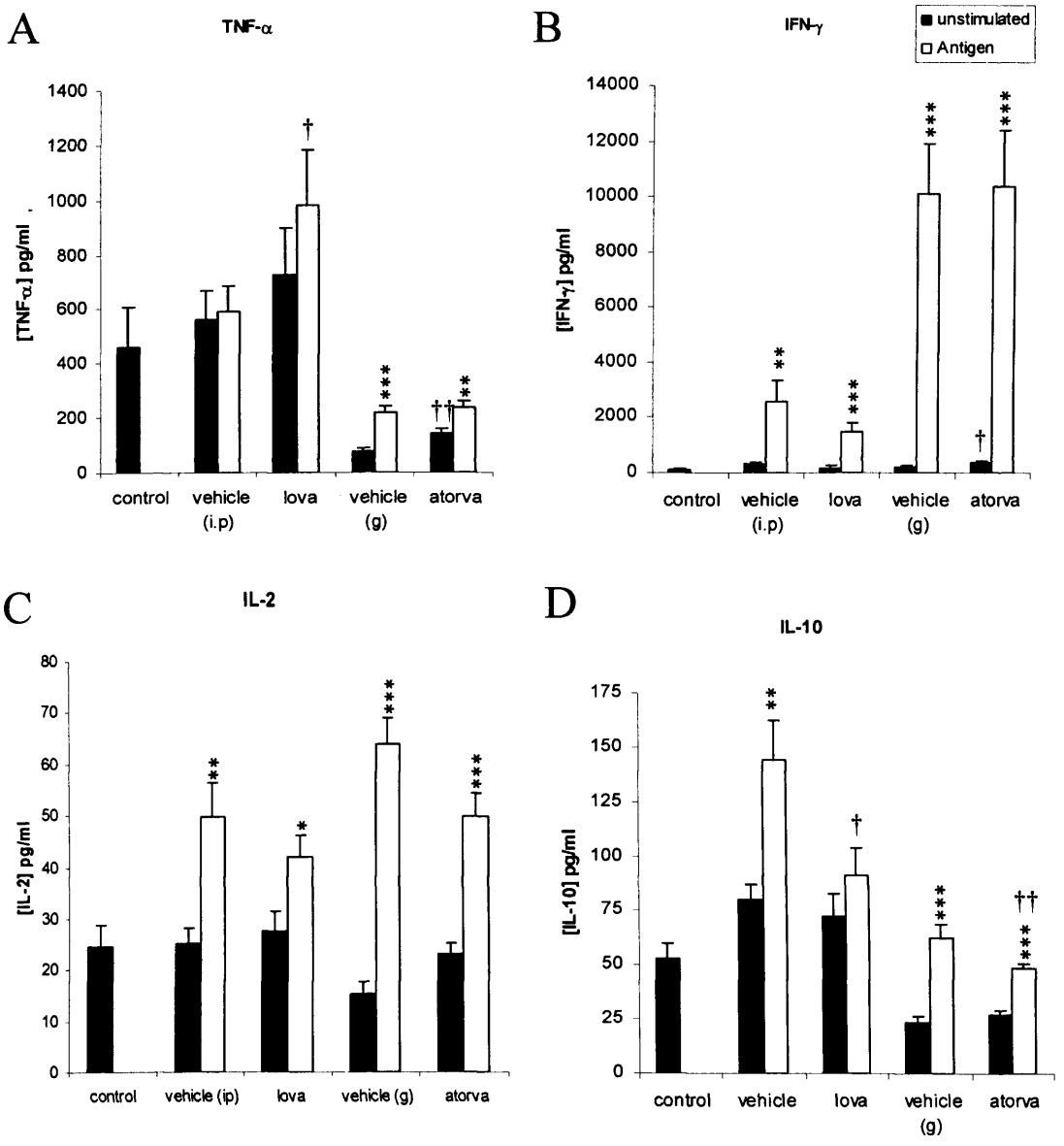

Figure 6.8 Statin treatment results in a significant increase in TNF-α production and a significant decrease in IL-10 production as compared with vehicle-treated splenocytes.

Atorvastatin therapy results in a small increase in TNF-α and IFN-γ and IL-10 production as compared to vehicle controls splenocytes. Splenocytes were cultured at a density of $5x10^6$/ml for varying time periods after which supernatants were harvested, stored at -70°C until assay for cytokine production by ELISA. Histograms show means ± SEM of duplicate wells from at least 6 animals from three independent experiments. Statistical analysis was determined using the Mann-Whitney U test. Statistical significance was determined as follows; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ as compared to unstimulated control and † $p \leq 0.05$ and †† $p \leq 0.01$ as compared with vehicle control. Control samples stimulated with antigen were not analysed.

-γ and IL-10 ($p \leq 0.05$) and an increase in TNF-α ($p \leq 0.05$) following antigen stimulation as compared to the corresponding vehicle group (Figure 6.8).

A significant increase in constitutive secretion of TNF-α was detectable in the atorvastatin treated group as compared with vehicle-treated splenocytes ($p \leq 0.01$). Stimulation of both vehicle gavage and atorvastatin treated splenocytes with IRBP peptide resulted in a significant increase in IFN-γ ($p \leq 0.001$), TNF-α ($p \leq 0.001$ and $p \leq 0.01$, respectively), IL-10 ($p \leq 0.001$) and IL-2 ($p \leq 0.001$). Atorvastatin treatment resulted in an increased constitutive secretion of TNF-α ($p \leq 0.01$) and a significant decrease in IL-10 in response to antigen as compared to vehicle controls ($p \leq 0.01$).

TGF-β cytokine secretion was assessed in vehicle treated and lovastatin treated splenocyte cultures (data not shown). Constitutive and antigen stimulated secretion of TGF-β by vehicle (i.p.) treated splenocytes was 525.5 ± 64.6pg/ml and 511.4 ± 49.4pg/ml whilst constitutive and antigen-induced TGF-β secretion by lovastatin treated splenocytes was found to be 512.6 ± 61.4pg/ml and 510.5 ± 42.1pg/ml respectively. No significant differences were detectable in the levels of TGF-β between each experimental group

In all samples assayed, very low levels of IL-4, IL-5 or IL-12 were detectable by ELISA. IL-4 and IL-5 secretion was also investigated by cytometric bead array and again no significant levels of IL-4 or IL-5 were detectable.

Statin treatment of EAU does not induce T regulatory cells

Previous studies have shown a clear switch from a Th1 cytokine profile to a Th2 cytokine profile as a result of statin therapy (Youssef *et al* 2002, Stanislaus *et al* 2002, Neuhaus *et al* 2003, Aktas *et al* 2003, Nath *et al* 2004). In contrast our results suggest that statins ameliorate clinical EAU in the absence of a Th1 to Th2 switch in the B10.RIII mouse.

We therefore set about to determine whether statin therapy may elicit any effects upon immunoregulatory T cells (Tregs). Tregs were identified by their co-expression of CD4, CD25 and CD62L (Fisson *et al* 2001). Flow cytometric analysis of $CD4^+CD25^+CD62L^{hi}$ cells showed no significant difference in the levels of phenotypically determined Tregs in vehicle, lovastatin or atorvastatin-treated groups (Figure 6.9).

A preliminary experiment to investigate the presence of functional Tregs was designed. In an attempt to assess whether the degree of clinical efficacy of statin treatment correlates with the modulation of Treg populations, PLN T cell populations from individual animals were tested for their ability to suppress vehicle-treated PLN T cell antigen-mediated proliferation.

$CFSE^+$ PLN T cells isolated from a vehicle-treated animal of grade 5 disease were cultured alone or in the presence of IRBP peptide [20μg/ml], with Con A [5μg/ml] as a positive control. Cells were also incubated with IRBP peptide in the presence of increasing numbers of PLN T cells isolated from a vehicle-treated animal of grade 4 disease, lovastatin-treated animals of grade 1 and 0 disease and atorvastatin-treated animals of grade 0, 0 and 4 disease. Proliferation of $CFSE^+PI^-$ vehicle treated PLN T cells was assessed at day 5 by flow cytometry. A low level of proliferation was observed in unstimulated control cultures (Figure 6.10 A) while maximal proliferation was observed in response to Con A (Figure 6.10 B). Proliferation was detected in antigen stimulated PLN T cells in the presence of putative Tregs (Figure 6.10 C).

Proliferation detected by CFSE can be quantitated following the method of Angulo and Fulcher (1998) and can be represented by the weighted division index. Quantitation of $CFSE^+PI^-$ responder PLN T cell proliferation showed that by day 5 a low level of proliferation was observed in unstimulated and antigen stimulated cultures, with marked proliferation detectable in response to Con A ($p \leq 0.01$; Figure 6.11). Significantly greater proliferation was detectable in each of the responder cell

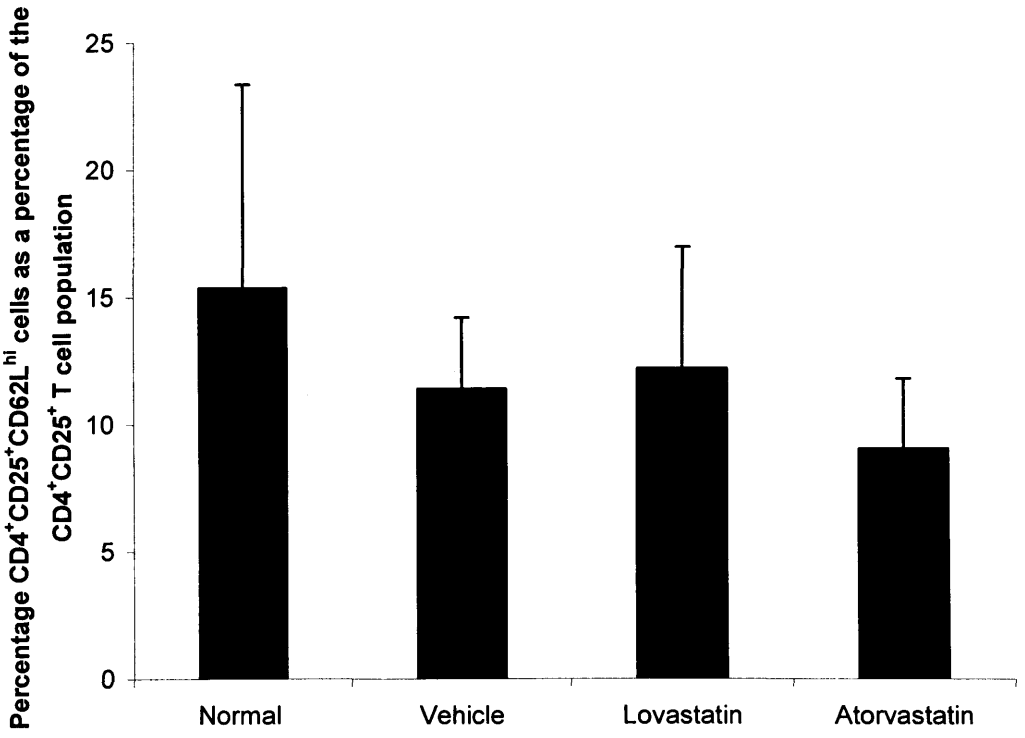

Figure 6.9 Statin therapy does not influence the level of $CD4^+CD25^+CD62L^{hi}$ cells as a marker of mouse Tregs.
Histogram shows the percentage of $CD4^+CD25^+CD62L^{hi}$ cells as a percentage of the $CD4^+CD25^+$ T cell population of PLN isolated from normal (n=2), vehicle-treated (n=5), lovastatin-treated (n=3) and atorvastatin-treated animals (n=6). Data is shown as the means ± S.D. from one preliminary experiment.

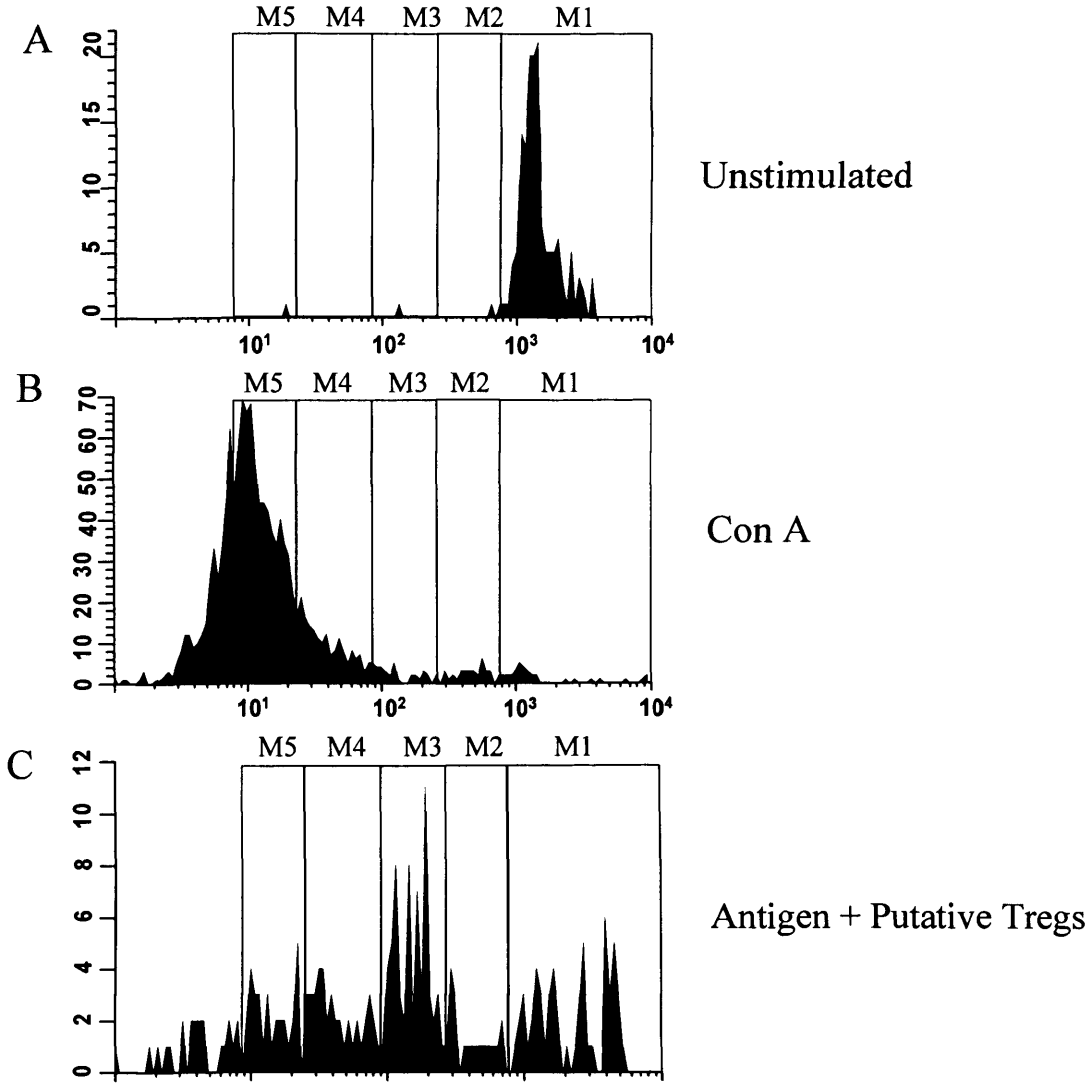

Figure 6.10 Representative histogram plots of CFSE$^+$ population peaks.
Panel A shows constitutive cell division within the unstimulated CFSE$^+$PI$^-$PLN responder T cell population. Panel B shows maximal proliferation of CFSE$^+$PI$^-$PLN T cells generated as a result of each cell division in response to Con A while panel C shows the reduction in CFSE fluorescence intensity generated as a result of cell divisions in response to IRBP antigen in the presence of putative regulatory T cells.

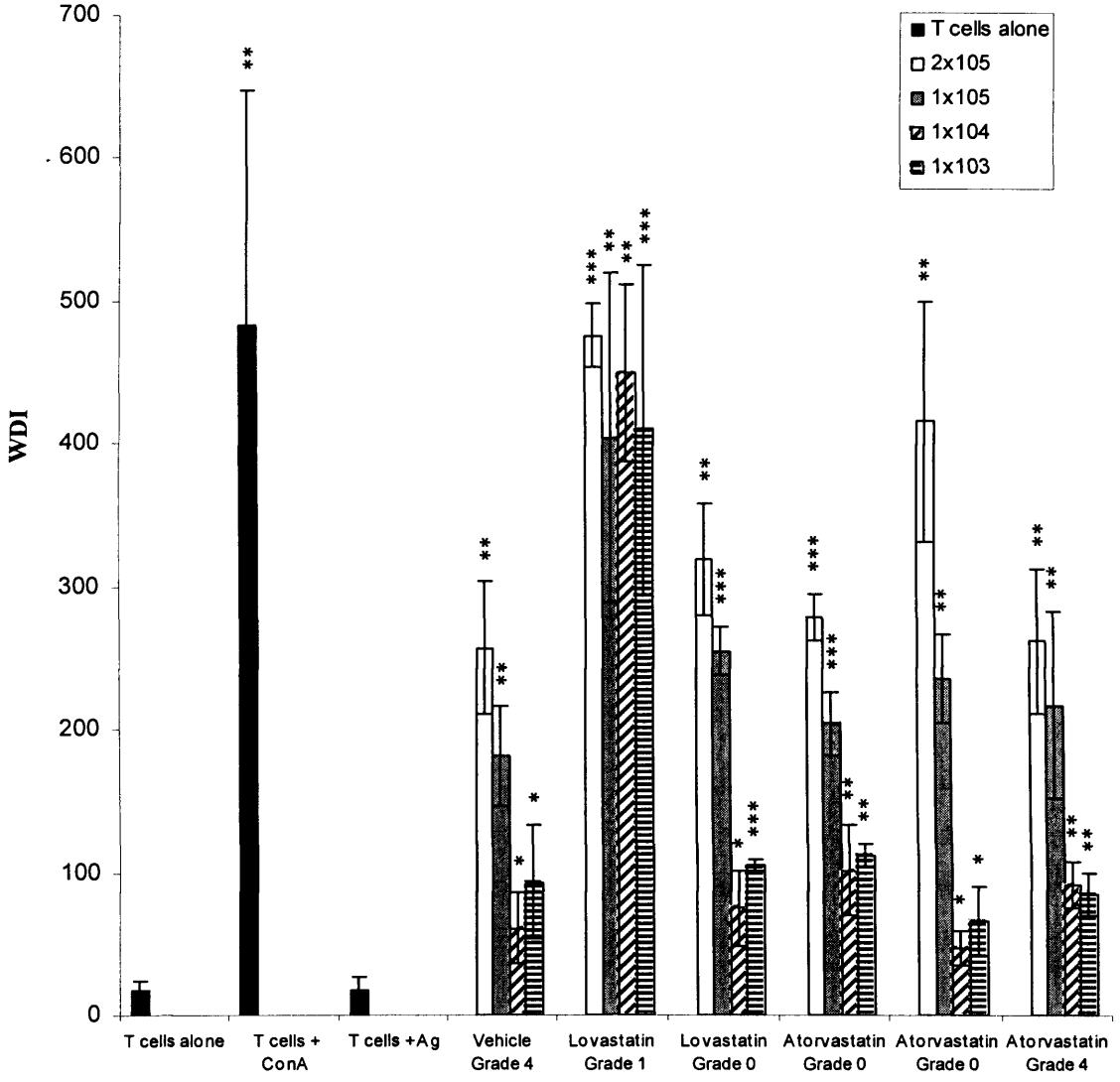

Figure 6.11 Introduction of vehicle, lovastatin and atoravstatin treated splenocytes to vehicle responder cultures results in increased proliferation of vehicle responder cells.

Histogram showing the weighted division index (WDI) of $CFSE^{+}PI^{-}$ PLN cells (responder cells) isolated from a vehicle treated animal and cultured alone, in the presence of ConA as a positive control or with IRBP peptide antigen in the presence or absence of increasing numbers of non-CFSE labelled syngeneic PLN cells isolated from different vehicle, lovastatin or atorvastatin treated animals as effector cells in an experiment in order to detect the presence of functional regulatory T cells. Proliferation of $CFSE^{+}PI^{-}$ PLN T cells was assessed at day 5 by flow cytometry. Statistical analysis was performed using the Students T test. *$p \leq 0.05$. **$p \leq 0.01$ and ***$p \leq 0.001$ as compared to antigen alone.

cultures cultured in the presence of increasing concentrations of effector PLN cells isolated from vehicle or statin treated animals ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$). The increase in proliferation observed as a result of the incorporation of putative Tregs cells does not support the presence of functional Tregs and no correlation with clinical efficacy of the statins *in vivo* was detectable.

Atorvastatin attenuates EAE in C57BL/6 mice but not EAU in B10.RIII mice

Our data clearly demonstrates that intraperitoneal administration of lovastatin results in the amelioration of clinical EAU, whilst oral administration of atorvastatin did not. We also found that neither lovastatin or atorvastatin treatment of EAU animals, resulted in the induction of a Th2 type cytokine profile. Given the findings by Youssef *et al* 2002 that intraperitoneal administration of atorvastatin ameliorates EAE by the induction of a Th2 cytokine profile, we set about to determine whether our findings were as a result of the therapy regimen we employed.

EAE was induced in C57BL/6 mice by active immunisation with MOG^{35-55} peptide as described by Youssef *et al* 2002 (see chapter 2 section 2.13.22). Atorvastatin therapy was initiated at day 10 (within one day of clinical symptoms) until the predicted peak of disease at day 17. Clinical scoring of EAE was performed following the criteria described previously from day 7 until peak disease (Youssef *et al* 2002). Clinical grading of atorvastatin-treated animals resulted in a significant reduction in clinical disease as compared with vehicle-treated controls between days 11 and 15 (Figure 6.12). Clinical grading of atorvastatin treated animals at day 16 and 17 post immunisation failed to reach significance when compared with vehicle treated controls.

Atorvastatin treatment of EAE but not EAU results in a Th2 profile

At peak of disease (day 17), splenocytes were isolated and levels of cytokine secretion determined within the culture supernatant at similar timepoints as described for EAU cultures (see chapter 2, section 2.13.26). Cytokine concentrations were again determined by ELISA. Antigen-stimulated IFN-γ and IL-2 secretion by splenocytes isolated from vehicle and atorvastatin-treated animals was significantly

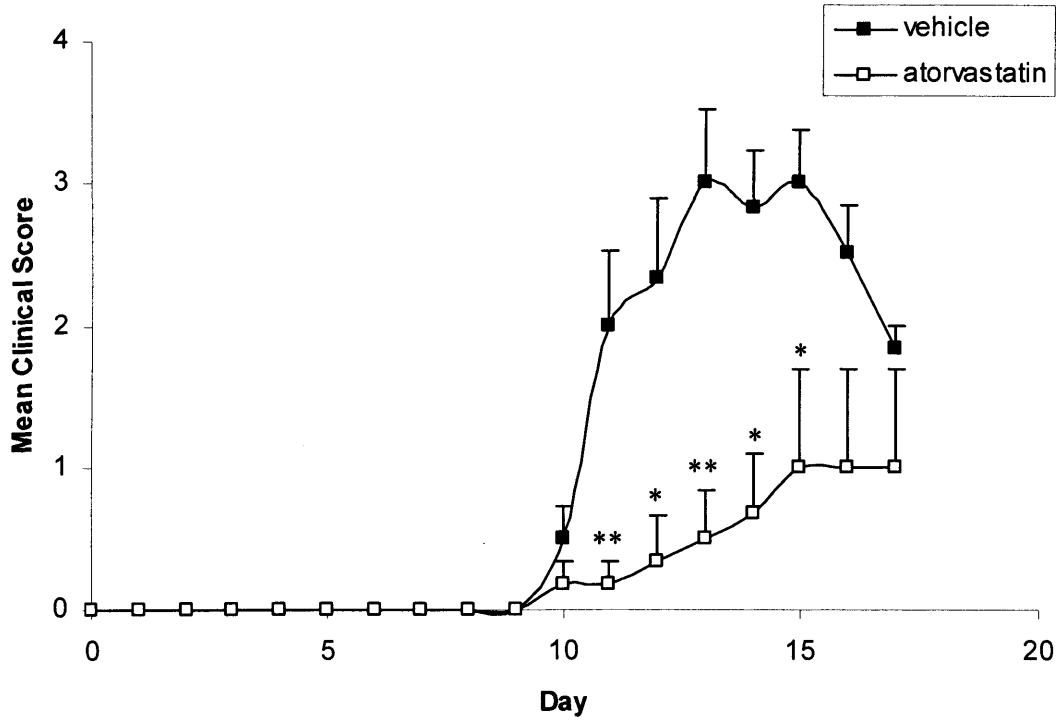

Figure 6.12 Atorvastatin treatment of MOGp35-55 induced EAE in C57BL/6 animals results in significant amelioration of EAE.
Atorvastatin was administered orally from day 10 post immunization of disease within one day of appearance of clinical symptoms. Clinical grading of disease was performed daily following criteria previously described (see chapter 2). Graph shows mean clinical score ± SEM. Statistical analysis was performed using the Mann-Whitney U test. * $p \leq 0.05$ and ** $p \leq 0.01$ as compared to vehicle controls.

increased as compared to basal secretion ($p \leq 0.001$ and $p \leq 0.01$ respectively). No significant difference in the level of basal TNF-α or in response to antigen was detectable in vehicle or atorvastatin cultures. Antigen stimulation of vehicle splenocyte cultures did not induce a significant increase in IL-10. However, a small but significant increase in IL-10 secretion was observed as a result of antigen stimulation of atorvastatin-treated splenocytes. Atorvastatin therapy resulted in a significant reduction in antigen-induced IFN-γ secretion ($p \leq 0.05$), a significant increase in constitutive IL-2 secretion and a significant reduction in both basal and antigen-induced secretion of TNF-α as compared with respective vehicle controls ($p \leq 0.05$ and $p \leq 0.001$ for unstimulated and antigen-stimulated cultures respectively). However atorvastatin therapy showed no significant effect upon IL-10 secretion by splenocytes. Production of IL-4 and IL-5 was again found to be below the sensitivity of detection.

Given the findings of Youssef *et al* 2002, in which a strong Th2 bias was observed in EAE after statin treatment we decided to investigate intracellular cytokine production by atorvastatin treated splenocytes from EAU and EAE animals. Intracellular cytokine staining of EAU splenocytes revealed very low levels of constitutive IL-4 staining (Figure 6.14) however antigen stimulation of atorvastatin-treated EAU splenocytes resulted in an increase in the IL-4 to IFN-γ ratio as a result of increased IFN-γ production (Figure 6.14) The constitutive IL-4 to IFN-γ ratio of atorvastatin-treated EAE splenocytes was found to be significantly lower than that of vehicle-treated EAE splenocytes, this was found to be as a result of increased IL-4 production (Figure 6.14). Antigen stimulation of atorvastatin-treated EAE splenocytes did not upregulate IFN-γ production as observed in EAU cultures and therefore presented with an IL-4 to IFN-γ ratio comparable with that of antigen-stimulated vehicle-treated EAE splenocytes (Figure 6.14).

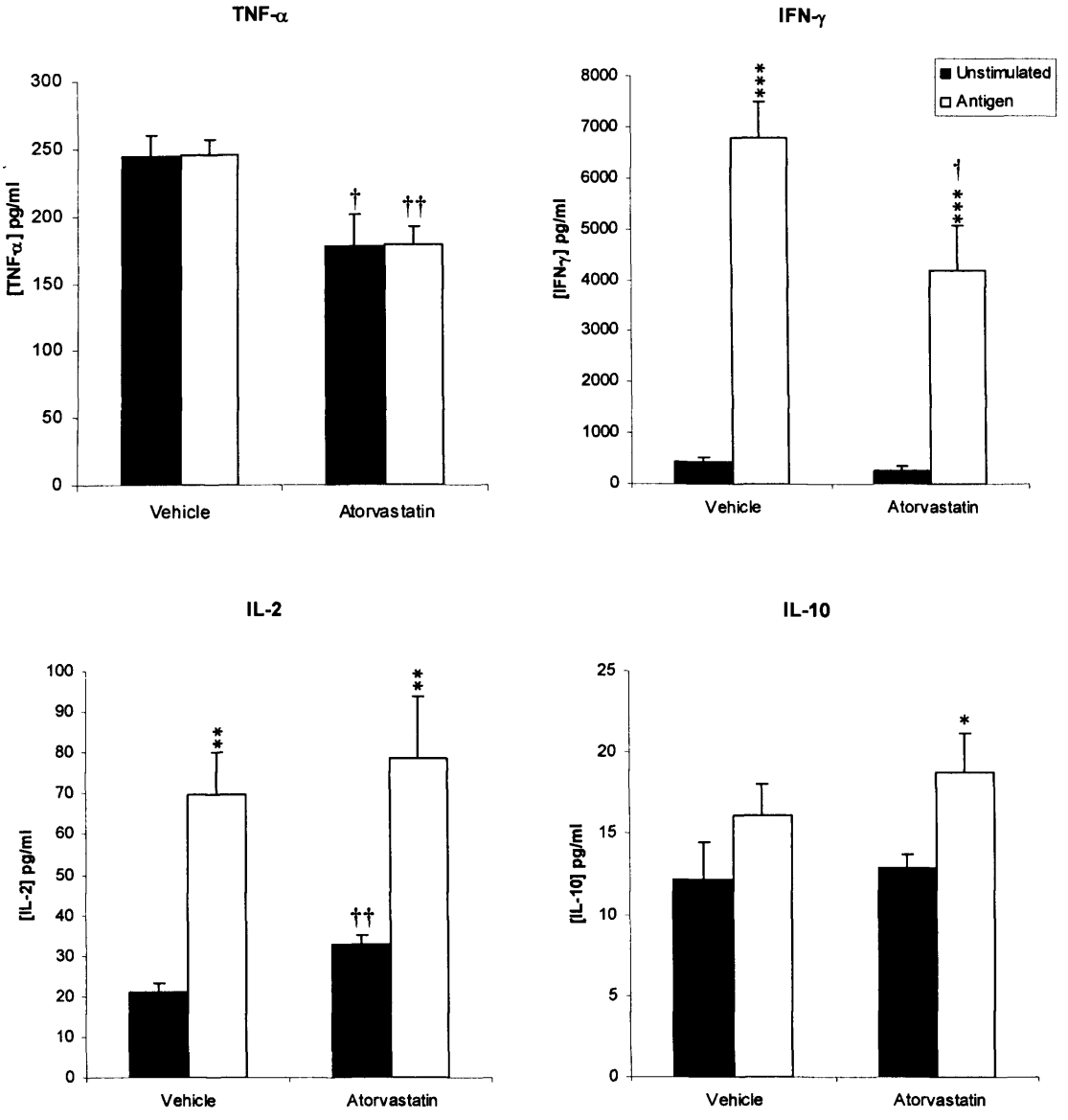

Figure 6.13 Atorvastatin therapy results in a significant decrease in TNF-α and IFN-γ and a significant increase in IL-2 production as compared to vehicle control.

Splenocytes were cultured at a density of $5x10^6$/ml for varying timeperiods after which supernatants were harvested, stored at -70°C until assay for cytokine production by ELISA. Histograms show means ± SEM of duplicate wells from at least 6 animals from one independent experiment. Statistical analysis was determined using the Mann-Whitney U test. Statistical significance was determined as follows; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ as compared to unstimulated control and † $p \leq 0.05$ and †† $p \leq 0.01$ as compared with vehicle control.

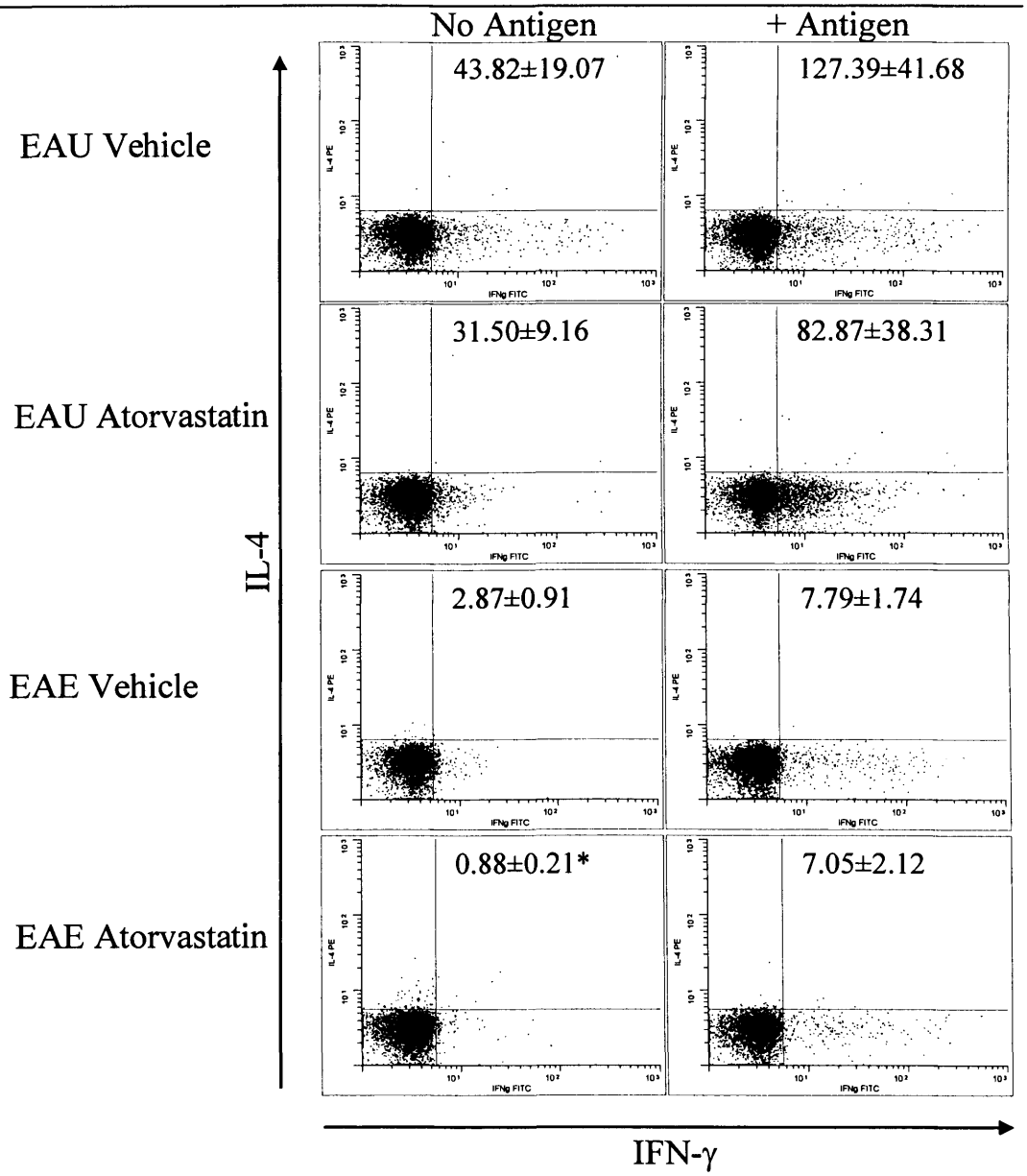

Figure 6.14 Representative dot plots showing constitutive and antigen-induced intracellular cytokine production by EAU and EAE PLN.
Atorvastatin therapy of EAE animals results in a reduced IFN-γ: IL-4 ratio (top right quadrant) as a result of increased IL-4 production. Atorvastatin treatment of EAU animals did not result in an increase in IL-4 production and antigen stimulation of atorvastatin cultures resulted in an increase in IFN-γ production. Intracellular cytokine staining for IFN-γ and IL-4 was performed on PLN T cells isolated from EAU animals and EAE animals at peak disease (days 12 and 17 respectively) as described in chapter 2 and analysed by flow cytometry. The IL-4 to IFN-γ ratio was calculated as the percentage of IL-4^+ cells as a ratio of the percentage of IFN-γ^+ cells. Data is shown as the means ± SEM of 6 individual animals from one independent experiment. Statistical analysis was performed using the Mann-Whitney U test. *$p \leq 0.05$.

Lovastatin acid but not atorvastatin acid is detectable in plasma from EAU animals

Plasma was harvested at expected peak of disease from EAU and EAE mice on days 12 and 17 respectively. Levels of circulating active atorvastatin and lovastatin in the form of atorvastatin acid and lovastatin hydroxyacid were determined (see chapter 2. section 2.13.18). No lovastatin hydroxyacid or atorvastatin acid was detected in the plasma of vehicle-treated EAU animals. Mean lovastatin hydroxyacid levels were found to be 0.098 ± 0.03μM (n=5) in plasma from lovastatin treated EAU animals and 0.101 ± 0.02μM (n=5) for lovastatin and squalene treated EAU animals.

Atorvastatin acid levels in EAU animals were found to be below the levels of detection of the assay (<0.1pM; n=16). In contrast, a low level of atorvastatin acid was detected in EAE plasma samples of 0.045 ± 0.03μM (n=6). These differences were observed despite following an identical dosing regimen for atorvastatin treatment of EAU and EAE animals.

Discussion

As a result of the findings presented in chapter 5, that lovastatin treatment of REC and RPE *in vitro* could prevent or inhibit lymphocyte transmigration across the BRB we set about to investigate whether statins were able to modulate lymphocyte trafficking *in vivo.*

Since statins have previously been shown to influence both lymphocyte TEM across a BEC monlayer (Greenwood *et al* 2003) and *in vivo* in EAE (MS; Stanislaus *et al* 2001, Youssef *et al* 2002, Greenwood *et al* 2003) and that the BBB and BRB share numerous properties it was therefore decided to test whether statins were able to influence clinical progress of a $CD4^+$ T cell-mediated autoimmune inflammatory disease of the retina. To test this hypothesis we first attempted to use a rat model of uveitis in the form of SAg peptide induced EAU (Fling *et al* 1991) however were unable to confirm clinical disease as measured by clinical grading, vascular disease and also retinal pathology at the timepoints and doses of peptide investigated. One possible explanation for this is that SAg peptide induced EAU exhibits a more delayed onset of clinical disease as compared with whole SAg induced EAU. Previous reports using SAg peptide did not address peak of disease and investigated disease after complete destruction of the retina between 20-28 days post immunisation (Fling *et al* 1991). It would therefore be worthwhile investigating the peak of SAg peptide induced EAU and to assess the effects of statins upon this model.

Due to our inability to induce reliable disease and our desire to test our hypothesis *in vivo* we decided to use an alternative mouse model. The model used for this purpose was IRBP peptide induced EAU in the B10.RIII mouse, as described by Hankey *et al* 2001. It is accepted that IRBP induced EAU in mice closely resembles that of SAg induced EAU in the Lewis rat (de Kozak *et al* 1981).

Subsequent studies using this mouse model revealed that intraperitoneal administration of lovastatin ameliorated clinical signs of EAU whilst oral

administration of atorvastatin failed to ameliorate clinical signs of EAU as assessed by established methods of grading of disease, such as clinical, vascular and retinal pathology. Previously it had been shown that oral, intraperitoneal and subcutaneous administration of atorvastatin prevented and alleviated symptoms of EAE (Youssef *et al* 2002, Stanislaus *et al* 2002, Greenwood *et al* 2003, Aktas *et al* 2003). Studies have also demonstrated that the alleviation of clinical EAE is associated with the induction of Th2 type cytokines (Youssef *et al* 2002, Stanislaus *et al* 2002, Aktas *et al* 2003, Nath *et al* 2004). Our own studies reveal that lovastatin treatment of EAU ameliorates ocular inflammation in the absence of a concomitant induction of a Th2 cytokine profile. This is in keeping with previous studies of statin treatment of collagen-induced arthritis (Leung *et al* 2004) and MS (Neuhaus *et al* 2002). Of interest is that it has previously been shown that remission from EAE is independent of IL-4 (Liblau *et al* 1997) which supports the role for statins in the amelioration of disease in the absence of a Th2 switch.

Our studies suggest that statin treatment of both EAU and EAE resulted in the inhibition of antigen induced cytokine production. However we investigated cytokine production by a mixed splenocyte population. Further work would therefore be essential to determine the source of the cytokines produced. Previous work by Nath *et al* (2004) showed that in their system the detectable Th2 profile was as a direct effect of statins upon T cells and not due to modulation of APC within a LN population. However, it is possible that once these cells are removed from their statin-containing *in vivo* environment, proliferation and cytokine production reverts to normal levels.

Previously, emphasis has been placed upon the ability of statins to ameliorate clinical disease by the suppression of Th1 type responses and the induction of Th2 type responses (Youssef *et al* 2002, Stanislaus *et al* 2002, Aktas *et al* 2003, Nath *et al* 2004). However a recent study by McKay *et al* (2004) has shown that statins are able to ameliorate clinical disease in a predominantly Th2 type disease. A reduction of Th2 type cytokines was observed in the absence of an increase in Th1 type cytokines.

This data therefore provides further evidence for the pleiotropic nature of statins and supports our hypothesis that amelioration of disease may occur independently of the induction of Th2 type cytokines.

Given the strong evidence for the induction of Th2 type cytokines, we investigated whether we were able to detect Th2 type cytokines induced by atorvastatin treatment of EAE following the method of Youssef *et al* (2002). Atorvastatin therapy resulted in significant amelioration of clinical disease but we were again unable to detect Th2 type cytokines by ELISA. Intracellular cytokine staining revealed a small increase in constitutive IL-4 production and hence a decrease in the IFN-γ: IL-4 cytokine ratio, which may be indicative of a Th2 type cytokine profile. In turn intracellular cytokine staining of atorvastatin treated EAU PLN T cells given IRBP *in vitro* failed to detect any increase in IL-4 production and therefore no indication of a possible induction of a Th2 profile. Given the degree of constitutive secretion of cytokines this would indicate that cells are relatively activated. The addition of antigen *in vitro* may then have resulted in tolerisation of Th2 secreting cells and therefore a suppression of IL-4 production.

One possibility for the inability to determine Th2 type cytokines may be due to the duration of statin therapy used in our studies. Cytokine production was investigated after 7 days of statin therapy in EAU animals, however previous studies investigated cytokine profiles after more than 12 days of statin therapy (Youssef *et al* 2002, Aktas *et al* 2003, Nath *et al* 2004). After investigation of Th2 type cytokine production at a number of timepoints (up to 120h) using cytometric bead array (CBA), ELISA and intracellular cytokine staining we can confidently say that IL-4 and IL-5 levels were below the sensitivity of detection in each of the assays employed. We would therefore suggest a number of possible explanations for this. Firstly, that increasing duration of statin therapy may contribute to the induction of a Th2 type profile or, secondly, that the B10.RIII mouse does not possess the capacity to produce Th2 type cytokines at the timepoints investigated. This latter possibility coincides with previous reports of extremely low-level production of IL-4 by B10.RIII mice in response to antigen, mitogen and superantigen (Johansson *et al* 2000, Tang *et al* 2000,

Xu *et al* 1997). Varying effectiveness of the ability of statins to induce Th1 or Th2 type cells has been determined *in vitro*. Hakamada-Taguchi *et al* (2003) demonstrated that lovastatin treatment of C57BL/6 splenocytes resulted in IL-4^+ Th2 type cells. This data would therefore suggest that our inability to detect Th2 type cytokines may be due to the strain of mouse used in our studies. Nevertheless our findings suggest that statin-induced amelioration of EAE is not simply due to the induction of Th2 type cytokines but that other mechanisms are involved.

In addition to the effects of statins upon cytokine production we investigated the effects of statins upon a number of additional splenocyte effector mechanisms. A significant observation was made upon harvesting of splenocytes at peak disease. Spleens isolated from lovastatin-treated animals were notably smaller than that of vehicle and control animals, which was reflected in the total splenocyte number. No such observations were made for atorvastatin treated animals. Given that vehicle-treated animals did not exhibit a decrease in splenocyte number as compared to normal controls, this would suggest the effects could be due to lovastatin and not as a result of the carrier solution in which lovastatin is administered. Failure to observe a decrease in splenocyte number as a result of vehicle gavage treatment would also suggest the decrease in splenocyte number to be independent of the mode of administration of the statin therapy. No other reports have described a role for statins in the modulation of splenocyte numbers. However our data suggests that lovastatin therapy impacts upon splenocyte development. It would therefore be interesting to investigate this further.

Our present study confirms that lovastatin inhibits $CD4^+$ T cell antigen-mediated proliferation which we were able to demonstrate by the incorporation of an anti-MHC class II antibody. Suppression of splenocyte proliferation by statins is consistent with that of a number of previous mouse studies (Youssef *et al* 2002, Aktas *et al* 2003, Leung *et al* 2004) and T cells in human studies (Hillyard *et al* 2002 and Neuhaus *et al* 2002). Lovastatin therapy resulted in decreased splenocyte proliferation over and above that of vehicle treatment alone, which was not observed as a result of atorvastatin treatment. This therefore highlights potential differences

between the statins employed. It is possible that differing dosing regimens may influence efficacy of treatment, given that differing mechanisms of metabolism or priming of effector cells may occur. This data would certainly suggest that inhibition of splenocyte proliferation is independent of therapy administration route.

In the absence of a detectable Th2 switch but significant reduction in disease incidence and severity we investigated whether lovastatin was able to influence clinical disease by the production of immunomodulatory T cells such as Tregs. We carried out preliminary studies to investigate Tregs through the expression of characteristic cell surface marker expression (Fisson *et al* 2003) and also assayed for the presence of functional regulatory T cells through the suppression of T cell proliferation (Stephens *et al* 2001, Taams *et al* 2001, Jonuleit *et al* 2001). Tregs have previously been shown to play an important role in the prevention of EAU (Takeuchi *et al* 2004). As a result of our present study we are able to confirm the presence, phenotypically, of Tregs at levels comparable with that of expected levels (Maloy and Powrie 2001, Fisson *et al* 2003). However, we were unable to determine the presence of functional Tregs, as assessed by the suppression of antigen-mediated proliferation. In contrast, we observed a significant increase in responder cell proliferation. This we believe is not due to allo-responsiveness given the inbred nature of the B10.RIII mouse and would therefore predict that the induced proliferation may be due to the highly activated nature of the statin-treated cells introduced to the cultures and cytokines which they may secrete. Possible reasons for our inability to detect functional regulatory T cells may be due to the low levels of regulatory T cells present within the cultures or due to the removal of the T cells from their *in vivo* statin-containing microenvironment. It has previously been shown that removal of T helper cells from an immunosuppressive environment results in the rapid restoration of T cell responses (Kupiec-Weglinski *et al* 1985). Secondly, impaired suppressive activity of Tregs has been implicated in the pathogenesis of human disease including MS (Viglietta *et al* 2004). Therefore it may be possible that our inability to determine functional Tregs is due to impaired function of Tregs in EAU. It would therefore be interesting in the future to investigate the effect of

exogenous lovastatin upon *in vitro* cultures. It would also be intriguing to investigate whether statin-treated PLN from EAU induced animals are capable of suppressing or preventing disease in naïve animals as has previously been described in a mouse model of EAE (Youssef *et al* 2002).

Unlike the serum cholesterol-lowering effects reported for statins in the treatment of humans, we found that lovastatin treatment of B10.RIII mice did not affect serum cholesterol. A small but significant increase in serum cholesterol was observed in atorvastatin treated animals as compared to normal controls. Previous reports have shown that statins have no effect upon serum cholesterol levels in mice (Chen *et al* 2003, Fischetti *et al* 2004, Naoum *et al* 2004). Treatment with lovastatin and squalene resulted in an increase in serum cholesterol, which may be explained by the presence of additional squalene, a key protein in the cholesterol synthesis pathway.

Co-administration of mevalonolactone with lovastatin resulted in a restoration of disease to levels towards that of vehicle control while administration of lovastatin and squalene resulted in the amelioration of EAU with effects comparable to those observed *in vitro* (Chapter 5). This data therefore strengthens the hypothesis that one possible mechanism of action of statins is the inhibition of isoprenoid groups essential for intracellular ICAM-1 signalling and subsequently lymphocyte migration across the BRB. These findings mirror that of results previously published from the laboratory, in which co-administration of mevalonolactone with lovastatin is able to restore clinical disease in a mouse model of EAE and restore migration across an *in vitro* model of the BBB (Greenwood *et al* 2003). This data is also in keeping with studies involving prenyltransferase inhibitors (Walters *et al* 2002). Other groups have shown statin therapy to result in an inhibition of inflammatory cell infiltrate in EAE (Youssef *et al* 2002, Greenwood *et al* 2003, Aktas *et al* 2003, Nath *et al* 2004) and also across non-CNS vascular endothelia, as demonstrated using *in vivo* models of inflammatory arthritis (Leung *et al* 2004) and allergic asthma (McKay *et al* 2004).

Of interest was the efficacy of lovastatin but not atorvastatin in the treatment of EAU and subsequently the clear amelioration of EAE but not EAU by atorvastatin therapy

administered following the same dosing regimen. We quantitated the statin levels within plasma obtained from lovastatin and atorvastatin treated animals. Analysis of the active acid form of lovastatin and atorvastatin revealed that treatment of EAU resulted in circulating active lovastatin hydroxyacid but not active atorvastatin acid in the B10.RIII mouse. Interestingly, atorvastatin treatment of EAE in the C57BL/6 mouse following the same dosing regimen resulted in low levels of circulating active atorvastatin acid. This data therefore would suggest clinical efficacy to be highly dependent upon genetic backgrounds of the hosts. This data highlights the possibility that levels of active circulating statin may depend upon the route of administration. Previous reports have suggested both the B10.RIII (Silver *et al* 1999) and C57BL/6 mouse (Sun *et al* 1997) exhibit a Th1 bias. In light of our findings, that low levels of circulating atorvastatin were detectable and also that the C57BL/6 mouse displays a greater propensity to produce Th2 type cytokines in comparison to the B10.RIII mouse. It is possible therefore that the C57BL/6 mouse is more amenable to atorvastatin treatment, especially if given orally.

This data clearly suggests the potential for lovastatin in the amelioration of clinical EAU by the inhibition of lymphocyte trafficking into the CNS and modulation of T cell function. This data, in conjunction with that presented in Chapter 5, provides strong evidence to suggest that the inhibition of lymphocyte trafficking is achieved by the specific inhibition of isoprenoids essential for functional signalling of ICAM-1. Clinical trials are currently underway to investigate statins in the treatment of MS (Vollmer *et al* 2004) although as our study has highlighted, efficacy of a given statin may vary between disease and also with genetic susceptibility. Nevertheless this data provides evidence to suggest that statins may offer the promise of an additional therapy in the treatment of sight-threatening posterior uveitis.

Chapter 7

General Discussion

General Discussion

The aim of this thesis was to investigate the role of CNS EC in the regulation of T lymphocyte function within the CNS and whether it would be possible to modulate lymphocyte entry, with a view to a potential therapy for the treatment of immune-mediated inflammatory diseases of the CNS such as MS and uveitis.

It is widely accepted that there are several specific mechanisms within the CNS designed to protect the delicate microanatomy of the CNS including the retina, which are actively involved in maintaining immune privilege through the suppression of potentially damaging immune responses (Griffith and Ferguson 1997). Both MS and uveitis are characterized by the presence of highly activated $CD4^+$ T lymphocytes within the CNS (Bellamy *et al* 1985, Deschennes *et al* 1988) with little or no inflammatory cell infiltrate observed within the CNS under normal conditions. Continuous surveillance of the CNS is known to occur by activated T cells (Hickey *et al* 1991) thus suggesting that during disease, mechanisms which regulate T lymphocyte function within the CNS is compromised.

In the initial studies I investigated the effects of TEM upon T cell function. It was found that highly activated T cells which migrated through BEC monolayers exhibited a significant increase in apoptosis as compared with control T cells and that the induction of apoptosis was found to be both cell-contact dependent and BEC specific. In contrast, similar studies using HEV monolayers demonstrated protection against apoptosis, which we feel may be consistent with the functional role of HEV EC within the peripheral lymph node (PLN; Girard and Springer 1995). This data therefore suggests the induction of apoptosis to be a specialised mechanism of protection for the CNS. Our data and that of others support a role for apoptosis within the CNS (Pender *et al* 1992, Griffith *et al* 1995, Jorgenssen *et al* 1998) and we believe that BEC may play an important role in this process, by the induction of apoptosis in infiltrating lymphocytes.

Given the need for direct cell-cell contact in BEC induced T cell apoptosis and the previously determined role for FasL in the induction of apoptosis within the eye (Griffith *et al* 1995) and its reported expression upon astrocytes in the normal rat brain as determined by immunohistochemistry (Bechmann *et al* 1999) we set about to investigate whether our BEC line expressed FasL. Unfortunately we were limited due to the paucity of antibodies for the detection of rat FasL and were unable to determine membrane bound FasL using the antibodies and techniques employed. However, RT-PCR analysis and analysis of BEC supernatants by ELISA would suggest FasL is expressed by BEC. This data supports the potential role of BEC in the specialised protection of the CNS from the immune system.

Treatment of BEC with pro-inflammatory cytokines such as IFN-γ and TNF-α resulted in the downmodulation of FasL mRNA levels and sFasL levels detected in BEC supernatants. Previous studies have suggested that conversion of membrane bound FasL to sFasL is a downmodulatory response (Suda *et al* 1997). Activated T cells within the CNS, especially during diseases such as MS and uveitis, are characteristically Th1 type cells which are capable of secreting large amounts of pro-inflammatory cytokines including IFN-γ. We would therefore propose that one mechanism by which T cells are able to persist in the CNS is through the downregulation of FasL expression by BEC. It is currently known that a number of human diseases including autoimmune lymphoproliferative syndrome arise through the dysregulation of cellular apoptosis (Lenardo 2003, Rieux-Laucat *et al* 2003). It is therefore plausible to suggest that the chronic inflammation which occurs during MS and uveitis may result from a downregulation of apoptosis. This is supported by reports of decreased expression of both Fas and FasL at the onset of EAE (Bonetti *et al* 1997) and increased apoptosis correlating with amelioration of disease in uveitis (Chan *et al* 1997, Dick *et al* 1999, Yu *et al* 1999), MS (Gniadek *et al* 2003), EAE (Pender *et al* 1992) and EAU (Sueda *et al* 2000, Abe *et al* 2001).

In light of our data, indirectly demonstrating FasL expression by BEC, it would be extremely interesting to demonstrate FasL expression by means of a functional assay.

Previous functional assays for the detection of FasL include the ability of FasL expressing cells to induce apoptosis in a Fas sensitive cell line (Rouvier *et al* 1993, Brunner *et al* 1995, Griffith *et al* 1995). This would be possible upon development of a cell line transfected with rat Fas which could be used in a bioassay based upon the principles of cytotoxicity that could be then measured by a CFSE and flow cytometry based assay. In the absence of a blocking antibody and the proposed downregulation of FasL mRNA by vitamin E it would be interesting to investigate the effect of pre-treatment of BEC with vitamin E as previously described (Li Weber *et al* 2002) upon T cell viability as a result of TEM using our *in vitro* assay.

ICAM-1 and ICAM-1 signalling at the BBB is pivotal to lymphocyte migration (Adamson *et al* 1999, Greenwood *et al* 2003, Greenwood *et al* 2003b, Lyck *et al* 2003). It has previously been shown that lymphocyte migration across the BBB can be modulated by the action of statins, which disrupt the intracellular signalling pathway of ICAM-1 in a mouse model of EAE (Greenwood *et al* 2003). As in EAE, a critical stage in the pathogenesis of EAU is lymphocyte migration into the CNS. We therefore set about to determine whether statins are able to disrupt signalling pathways within cells of the BRB.

In vitro inhibition of lymphocyte migration by the specific inhibition of the ICAM-1 signalling pathway in both REC and RPE monolayers by statin induced depletion of isoprenoid intermediates was detected. *In vivo* we also demonstrated that statin treatment results in reduced incidence and severity of clinical EAU. We therefore propose that inhibition of lymphocyte migration may contribute to the amelioration of clinical EAU.

In addition to the inhibition of lymphocyte migration, we describe a number of additional effects of statins which will undoubtedly play a role in the amelioration of EAU, including modulation of T cell proliferation and cytokine production which is consistent with that of reports for statin treatment of EAE (Youssef *et al* 2002, Stanislaus *et al* 2002, Aktas *et al* 2003, Nath *et al* 2004). However, in contrast to that

of the effects of statins in EAE, we were unable to confirm polarisation of cytokine profiles towards a Th2 type profile (Youssef *et al* 2002, Stanislaus *et al* 2002, Aktas *et al* 2003, Nath *et al* 2004). We can report suppression of antigen-mediated cytokine production and proliferation by the statins, as has been described by others, and believe that a decrease in pro-inflammatory cytokines which are known to exacerbate disease may aid in the resolution of disease. It is possible that this decrease in pro-inflammatory cytokines may result in decreased expression of adhesion molecules at the level of the BRB, which are essential for T cell infiltration. This may therefore result in the decreased activation of circulating T cells and subsequent recruitment into the CNS.

Given the highly pleiotropic effects of statins and the knowledge that statins are able to modulate EC function, I would be extremely interested to investigate whether statins influence FasL expression at the level of the BRB. Given our theory that FasL expression is decreased during onset of disease and that resolution is associated with a restoration in apoptosis, I would like to determine FasL expression within the CNS of statin treated animals to determine whether resolution of disease is associated with statin induced upregulation of FasL. It would also be interesting to investigate the effects, if any, of systemic statin treatment upon the CNS. Studies to date have investigated peripheral responses, even though it is known that statins are able to penetrate the BBB (Guillot *et al* 1993, Saheki *et al* 1994).

Our *in vivo* studies highlight a number of factors, which must be considered in the treatment of disease. A major implication for the use of statins in the treatment of human uveitis includes the fact that patients with posterior uveitis will present to the clinician with ongoing chronic inflammatory disease. Our *in vivo* studies address lovastatin therapy in a prophylactic manner. Statin treatment of EAE has been investigated upon onset of clinical symptoms and during acute disease (Youssef *et al* 2002, Greenwood *et al* 2003, Aktas *et al* 2003) and revealed clinical efficacy upon administration at onset of clinical symptoms, but not in established EAE (Nath *et al* 2004). However lovastatin has been shown to prevent relapse in a relapsing remitting

model of EAE (Greenwood *et al* 2003). Our present studies investigated the effects of lovastatin in an acute model of EAU. It is widely known that upon total destruction of the retina disease spontaneously resolves. This is in contrast to the relapsing nature of many EAE models in which remyelination of the CNS is known to occur in MS (Brűck *et al* 2003). It would therefore be interesting to assess the effects of statins upon active EAU disease as this may determine the value of statins in the treatment of uveitis and also in a more chronic inflammatory disease model.

Our study also raises the question of whether efficacy of a statin is dependent upon the disease to be treated. We show clear amelioration of EAU with lovastatin but not with atorvastatin therapy, while numerous independent studies reveal amelioration of EAE by atorvastatin. This therefore highlights a major factor for consideration. Although the effects observed may be as a result of bias generated through the use of susceptible animal strains for an *in vivo* model, which we feel may partially account for the absence of a Th2 switch in the B10.RIII mouse, it has previously been shown that treatment efficacy in MS may not correlate with efficacy in uveitis and *vice versa.*

Of great importance in the acceptability of a drug treatment is the ease and mode of delivery. Ideally treatment should be well-tolerated orally. Stains are given orally and are generally well tolerated (Baker *et al* 2003). However some reported side effects of statins include exercise intolerance, myalgia and myoglobinuria, which are thought to be due to statin induced inhibition of Co-enzyme Q10 (CoQ10; Rundek *et al* 2004) which may be rectified by dietary intervention or CoQ10 supplements (Pettit *et al* 2003). Clinical trials are currently underway for oral simvastatin in the treatment of MS with promising results (Vollmer *et al* 2004). One report provides compelling evidence for the use of statins in children with familial hypercholesterolemia, with no detectable effects upon growth, muscle or liver enzymes or endocrine function (Wiegman *et al* 2004).

Current therapies in MS and uveitis aid in the management of disease. Removal of immunosuppressive therapy such as cyclosporine or prednisone from transplant recipients results in rejection of the organ (Kasiske *et al* 2000) and it has previously been shown that removal of statins results in recurrence of EAE (Greenwood *et al* 2003). Statin therapy therefore can only be considered as a form of management of disease but with considerably fewer side-effects than other drugs available, and if steroid-sparing, would be an attractive option. This data is the first known report of statins in the treatment of EAU and provides evidence for the potential of statins in the treatment of EAU as a monotherapy or as an element of combination therapy.

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Appendix 1

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FasL-mediated apoptosis induced by CNS endothelial cells

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T-cell apoptosis as a mechanism of immune privilege

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